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13. ABSTRACT

We theorize that ß-lapachone is an effective agent against prostate cancer (CaP) cells/tumors. It kills cells by targeting elevated NQ01 levels in CaP tissue. ß-Lapachone targets ionizing radiation (IR)-inducible NQ01. Normal cells with little or no NQ01 levels are spared, while IR-treated CaP cells expressing NQ01 are killed by apoptosis. ß-Lapachone killing of CaP tumors is independent of p53, pRb, cell cycle status, or hormone (non)responsiveness and involves calpain activation. We are testing this theory using NQ01-deficient LNCaP cells, and comparing these cells to constitutively expressing NQ01 cells, with or without inhibitors of the enzyme (i.e., dicoumarol). We are examining whether constitutive or inducible NQ01 levels are required for radiosensitization by ß-lapachone. In Aim 1, *in vitro* responses and mechanisms of cell death after ß-lapachone are examined, with or without IR, using cultured human CaP cells. In Aim #2, we will examine the efficacy of radiosensitization by ß-lapachone *in vivo* using human CaP xenografts in male nude mice. Preclinical animal studies to move ß-lapachone, from the bench to clinical trials against CaP have started. Three routes of ß-lapachone administration were developed:

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FOREWORD

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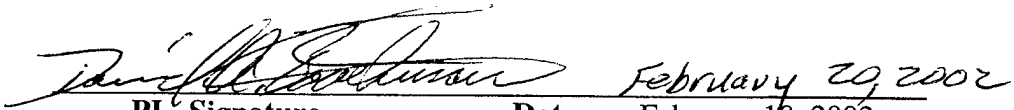

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INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose, and scope of the research.*

ß-Lapachone, an ortho-naphthoquinone found in the bark of the South American Lapacho rainforest tree, appears to be a promising agent for the treatment of prostate cancer (CaP) cells. CaP is a disease of slow growing neoplastic cells. Agents that kill CaP cells independent of cell cycle status or hormonal regulation are needed. ß-Lapachone is a novel apoptotic inducing agent that: **(a)** kills CaP cells independent of androgen-response, p53 or pRb status, and cell cycle phase; quiescent CaP cells are killed as effectively as cycling cells; **(b)** causes lethality only in CaP cells expressing NADP(H):quinone oxidoreductase (NQ01), an ionizing radiation (IR)-inducible two-electron reductase that is also elevated in many human CaP cells; and **(c)** causes an unique form of apoptosis in CaP cells that is caspase-independent and mediated by activation of calpain or a calpain-like protease. Since CaP may be a disease of abnormal (i.e., blocked) apoptosis, rather than altered proliferation (Wertz and Dixit, J. Biol. Chem. 275: 11470-11477, 2000), ß-lapachone could be an agent ideal for this disease.

ß-Lapachone was originally discovered for its ability to radiosensitize a variety of human cancer cells. Without knowledge of its mechanism of action, however, the compound remained unexplored for use against CaP. Recent elucidation of the mechanism of action of ß-lapachone, showing that this compound is specifically activated by X-ray-inducible NQ01, now allows us to explore the preclinical potential of ß–lapachone (or more efficient analogs), with or without IR. **We hypothesize that ß-lapachone is an effective agent against CaP cells due to its ability to kill cells by a target which is elevated in CaP tissue. Furthermore, the compound will be even more effective in CaP tissues that up-regulate its activating enzyme, the IR-inducible NQ01/xip3 oxidoreductase enzyme. Normal cells with little or no NQ01 levels will be spared, while CaP cells expressing NQ01 (or which induce NQ01) will be killed by a novel form of apoptosis induced by ß-lapachone futile cycling. CaP cell killing will be independent of p53, pRb, cell cycle status, or hormone (non)responsiveness.** We will test this theory using LNCaP cells that are deficient in NQ01, and compare their responses to cells constitutively expressing NQ01 (i.e., DU145, PC-3 and NQ01-transfected, LNCaP cells) in the presence or absence of inhibitors of NQ01 (e.g., dicoumarol). We will examine whether constitutive or inducible NQ01 levels are required for radiosensitization by ß-lapachone using conditional (doxycycline- or ecdysone-inducible) NQ01 expressing stable cell lines of LNCaP. In Specific Aim 1, we will examine *in vitro* responses and mechanisms of cell death after ß-lapachone, with or without IR, using human CaP cells in culture. In Specific Aim #2, we will examine the efficacy of radiosensitization by ß-lapachone *in vivo* using human CaP xenografts in male nude mice. Our studies should provide needed preclinical mechanistic data to move ß-lapachone, or one of its analogs, from the bench to clinical trials against CaP in the next three years. Such phase I studies with ß-lapachone can be performed with or without IR.

BODY OF GRANT UPDATE: *This section shall describe the research accomplishment associated with each Task outlined in the approved Statement of Work. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the annual/final report. However, appended publications and/or presentations MAY be substituted for a detailed description but MUST be referenced in the BODY of the report. If applicable, for each Task outlined in the Statement of Work, reference appended publications and/or presentations for details of result findings and tables and/or figures. The report shall include negative as well as positive findings, and also shall include any problems in accomplishing any of the tasks. Statistical tests of significance shall be applied to all data whenever possible. Figures and graphs referenced in the text shall be appended. The discussion shall include the relevance to the original hypothesis. Recommended changes or future work to better address the research topic may also be included, although changes to the original statement of work must be approved by the Grants Officer.*

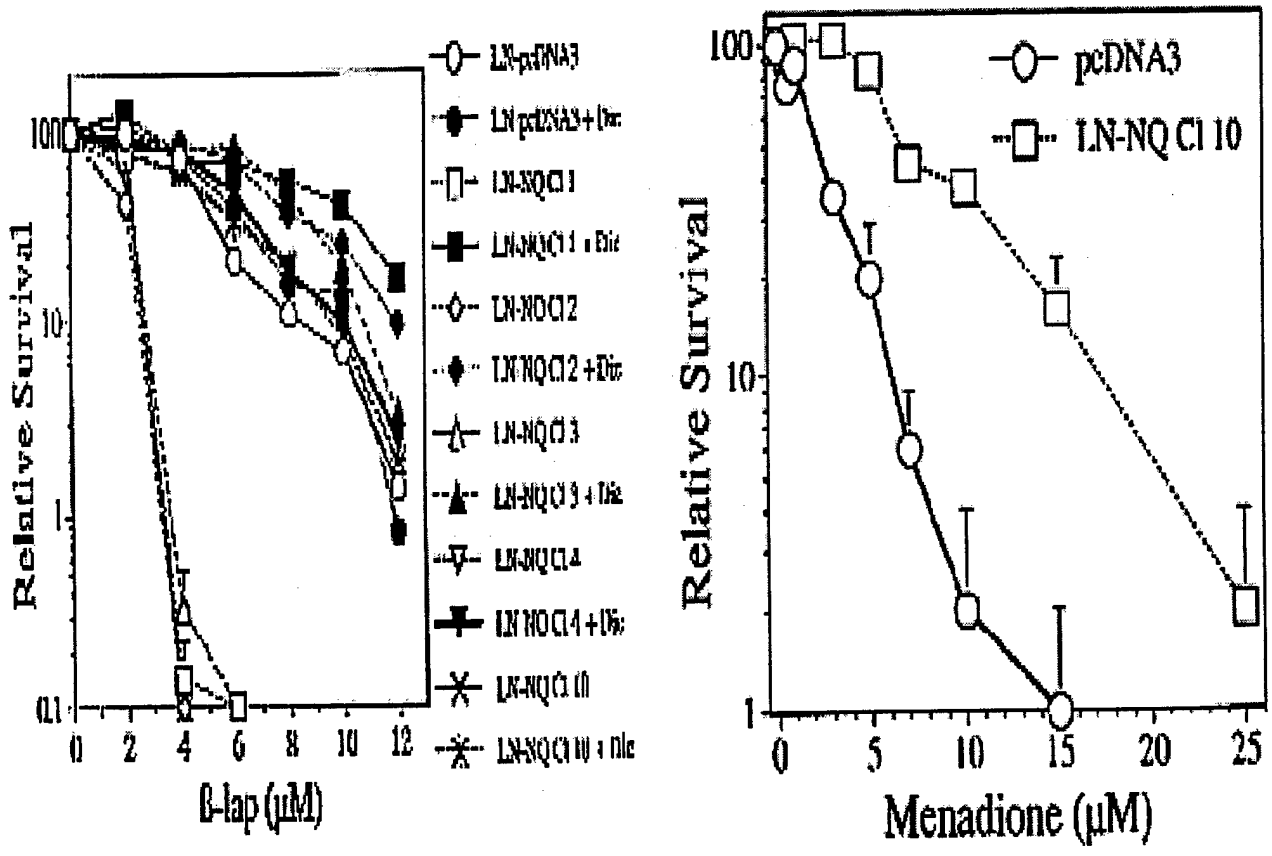
F: **Previous *Statement of Work and Accomplishments Made.***

Task 1: **Investigation of whether inducible or constitutive NQ01 levels are required for the ability of ß-lapachone to radiosensitize human prostate cancer (CaP) cells (Years 0-3).**

1. Develop constitutive NQ01-expressing LNCaP cells in culture.

Progress: We have established and published LNCaP cells that constitutively express NQ01 and demonstrated that they are responsive to ß-lapachone, whereas, vector alone expressing LNCaP cells are not responsive to the drug. These results were published in our recent paper in Experimental Cell Research (Planchon et al., Appendix): Figure 2 shows how all of the NQ01-expressing cells were killed by ß-lapachone, whereas, vector-alone cells were spared. Furthermore, we demonstrate that NQ01-expressing cells are spared from ß-lapachone cytotoxic responses by coadministering dicoumarol, a specific inhibitor of NQ01.

Fig. 2. **NQO1 enhances ß-lapachone, but decreases menadione, cytotoxicity.** Left, NQO1-containing (LN-NQ Cl1-4, 10) and -deficient (LN-pcDNA3) LNCaP clones were treated with 4-h pulses of various doses of ß-lapachone, ± 50 µM dicoumarol. Colony forming ability assays (CFAs) were performed three times, each in triplicate. Open symbols: ß-lapachone alone; Closed symbols: ß-lapachone + 50 µM dicoumarol. Right, one NQ01-transfected LNCaP clone (LN-NQ Cl 10) and the LNCaP vector alone clone (LN-pcDNA3) were treated with 4-h pulses of various doses of menadione and CFA assays were determined [45,60].



2. Establish repressor-expressing (either ecdysone- or tetracycline-inducible repressor), stably (neo resistant) transfected LNCaP cells. Isolate and test clones using doxycycline- or ecdysone-responsive luciferase reporter transfection assays ***(Months 0-3).***

Progress: We have developed tetracycline-inducible LNCaP cells that induce TRE-luciferase >50-fold after various doses of tetracycline. We are now constructing TRE-NQ01 for transfection and isolation of conditional-inducibility of NQ01 via administration of tetracycline.

3. Establish doubly transfected (neomycin and hygromycin) LNCaP clones from "1a" that conditionally express NQ01 in response to gradient doses of doxycycline or ecdysone. NQ01 levels under increasing conditional expression will be tested using Western blot analyses for NQ01 protein levels, as well as for NQ01 enzyme levels using a standard reduction assay. ***(Months 2-4).***

Progress: We are currently at this step. Steps 4-9 will be completed during this second year.

4. Clones from "1", as well as cells which have constitutive endogenous NQ01 levels (DU-145 or PC-3), will then be treated with ß-lapachone or menadione in the presence or absence of dicoumarol, or other NQ01 inhibitors. NQ01 protein and enzyme levels will then be monitored. ***(Months 2-4).***
5. Cells from "2" with then be treated with IR, +/-ß-lapachone, +/-gradient doses of dicoumarol, or +/- gradient doses of BAPTA-AM for 4h post-IR; BAPTA-AM is a calcium chelator that indirectly prevents ß-lapachone toxicity by preventing calpain or a calpain-like protease from being activated. Dicoumarol inhibits NQ01 and prevents ß-lapachone activation. IR responses will be compared to drug alone. Survival, apoptosis (caspase activation, TUNEL, apoptotic death substrate cleavage events, and pRb dephosphorylation assays), and alterations in cell cycle checkpoints will be examined; no alterations in checkpoints have yet been noted with this compound, but this information will be gathered by flow cytometry when examining apoptosis +/- TUNEL staining ***(Months 4-12).***
6. Menadione treatments will then be compared to ß-lapachone. Responses for the two drugs should be opposite. ***(Months 4-12).***
7. U1-Mel cells, a malignant melanoma cell line known to induce NQ01 after IR, will be investigated as a positive control; we originally cloned NQ01 as x-ray-inducible protein #3 from these cells and followed the induction kinetics of NQ01 transcript levels after IR exposure. ***(Months 0-12).***
8. Evaluation of role of NQ01 in the survival of CaP cells following IR independent of ß-lapachone exposures. ***(Months 4-12).***

Task 2: **Role of NQ01 in ß-lapachone-mediated radiosensitization *in vivo*.**

1. Establish xenografts in male nude mice containing androgen-slow release capsules from transfected as well as non-transfected LNCaP cell lines expressing constitutive (DU-145, PC-3, LNCaP-NQ clones (Fig. 2), or conditionally-inducible (doxycycline- or ecdysone-responsive) NQ01 levels. ***(Months 0-24).***
2. Western blot, enymology and immunohistological evaluation of NQ01 levels before and after various doses of doxycycline administered orally or i.p, will then be given to evaluate the time-course and dose-response induction of NQ01 levels in LNCaP transfected xenografts. ***(Months 8-24).***
3. Western blot, enzymology and immunohistological evaluation of NQ01 levels before and after dicoumarol administration orally or i.p. will be evaluated in time-course and dose-response experiments using DU-145 and PC-3 xenografts. (Months 0-12).
4. ß-Lapachone dose-response and time-course studies. Pharmacokinetics of the active intact parental form of the compound will be evaluated in blood, as well as in normal and prostate tumor tissues, as described by our laboratory. ***(Months 0-12).***
5. Efficacy of ß-lapachone given alone against xenografts derived from LNCaP transfected, as well as nontransfected and vector alone CaP cells, with or without doxycycline administration. ***(Months 8-24).***
6. Efficacy of ß-lapachone alone against DU-145 or PC-3 cells with or without dicoumarol administration. ***(Months 0-12).***
7. Efficacy of IR alone against DU-145 or PC-3 and LNCaP xenografts. (Months 0-24).
8. Efficacy of IR + ß-lapachone with or without doxycycline administration against LNCaP xenografts. Dicoumarol will also be used after a series of pilot experiments testing the influence of dicoumarol on doxycline, IR or ß-lapachone exposed mice. ***(Months 24-36).***
9. Antitumor, as well as morbidity and mortality evaluations, will be performed in steps 5-8 as described in Boothman et. al., Cancer Res. 47: 2344-2353, 1987; Cancer Res. 47: 2354-2362, 1987. ***(Months 8-36).***
10. Evaluation of apoptotic endpoints in normal compared to CaP tissue will then be evaluated. Caspase and calpain (specific cleavage as well as enzymological assays) activation assays will be performed along with ATP loss and PARP and lamin B cleavage reactions. Antibodies to activated calpain, caspases, cleaved PARP and lamin B are now available. The NQ01-dependent futile cycling of ß-lapachone in vivo will be tested, which results in dramatic loss of ATP and activation of calpain-like proteolysis. See J.J. Pink et. al., J. Biol. Chem., 275(8): 5416-5422, 2000 and Exp. Cell. Res., 255 (2): 144-155, 2000. ***(Months 0-36).***

Progress on task #2: Our progress on Aim #2 has been focused on developing delivery systems for ß-lapachone and beginning our first drug delivery of ß-lapachone into mice. Simultaneously, we have begun our first animal studies, and have started growing LNCaP xenografts in male nude mice.

Development of Drug Delivery Methods. A major limitation of the use of ß-lapachone has been in our inability to deliver the drug into animals in a way in which the drug is bioavailable. Previous *in vivo* studies by Li and Pardee et. al., PNAS, 1999, suggested that an optimal ß-lapachone administration dose was ~150 mg/kg. We suspect that this dose represents a significant amount of drug that was never bioavailable. Thus, we sought to develop new methods of drug delivery. For brevity, only the use of ß-cyclodextrins will be presented in this first update. In our next update, we will summarize two additional routes of drug administration: millirods and microspheres, both made from polymers that will be described in the next update.

New Methods of Drug Delivery:

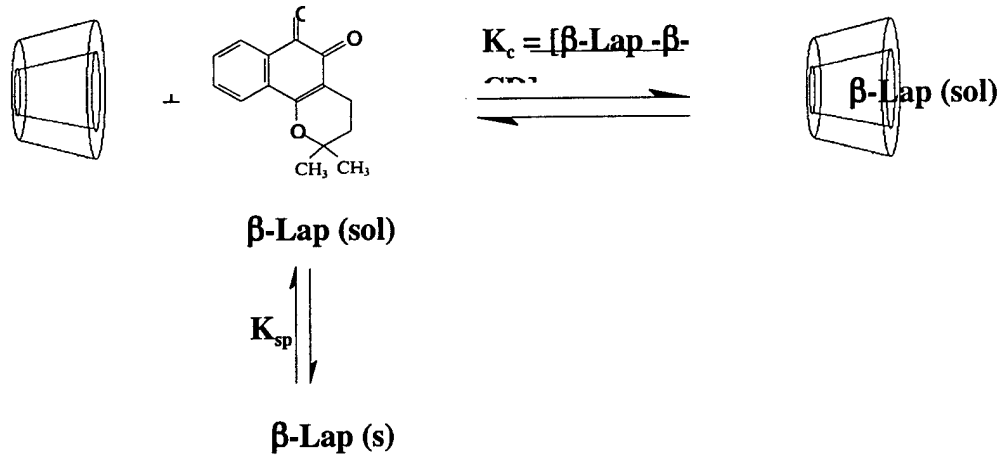
In collaboration with Dr. Jinming Gao, we developed three new methods of drug delivery: millirods, microspheres and ß-cyclodextrin.

ß-Cyclodextrin: Cyclodextrins (CDs) are well-known host molecules, which can form the inclusion complex with a variety of drugs to improve properties of drugs, such as solubility, stability and bioavailability. The α-cyclodextrin (α-CD) comprises six glucopyranose units, β-cyclodextrin (β-CD) comprises seven such units, γ-cyclodextrins (γ-CD) comprises eight such units and hydroxypropyl-beta-cyclodextrin is the modified β-cyclodextrin obtained by treating a base-solubilized solution of β-cyclodextrin with propylene oxide. The entire glucose unit are in the chair conformation linked by α(1,4) glycosidic oxygen bridges, which form a hydrophilic outer surface and a hydrophobic cavity. The hydrophilic outer surface with free hydroxy groups can make it water-soluble. The hydrophobic cavity can enhance the solubility of β-Lap by encapsulation or forming inclusion complexes in aqueous solution. In general, the main driving force for the inclusion complex between a poorly soluble guest and a CD in aqueous solution are the repulsive forces between the included water molecules and the apolar CD cavity and between the bulk water and the apolar guest.

During complex formation, β-Lap will be fit into CD cavity and establishes the dynamic equilibrium with free drug and CD molecules in the solution expressed by the complex association constant (K_c). For the formation of a 1:1 complex, K_c and the equilibrium are shown in Figure 1.

The aim of this work was to explore the use of cyclodextrins to form inclusion complex with β-Lap to overcome the solubility problem of this compound. The effects of CDs on the aqueous solubility of β-Lap and the interaction of CDs and β-Lap were studied by UV and fluorescence spectrometry, which can provide the association constant (K_c). In addition, the effect of the inclusion complex is evaluated on cell cytotoxicity in DNA assay.

Figure 1. **β-Lap structure and schematic diagram of the equilibrium of β-Lap in aqueous solution in the presence of CD where [CD-β-Lap] is the concentration of 1:1 CD-β-Lap complex, [β-Lap] is the concentration of free β-Lap and [CD] is the concentration of free CD in the equilibrium.**



Materials- Alpha, beta, gamma and hydroxypropyl-beta-cyclodextrin were obtained from CTD, Inc., which is over 98% purity. β-Lapachone was obtained from Boothman 's lab. All chemicals were used without further purification.

Dissolution studies of β-CD-β-Lap inclusion complex- Dissolution studies were performed by adding an excess amount of β-Lap (4 mg) in 4 ml of 20 mM potassium phosphate buffer (pH 7.7) containing 0 and 1.58×10^{-2} mol/l of β-CD then these solution were stirred at 25 °C. An aliquot was taken at intervals; from its filtrates the amount of β-Lap was determined by UV spectrophotometry (Perkin Elmer instruments Lambda 20) at the wavelength of 257.2 nm.

Phase Solubility studies- Solubility studies were performed by adding an excess of β-Lap in PBS containing different amount of CDs ranging from 0 to the highest solubility of each CD, and stirred at 25 °C until equilibrium. An aliquot was withdrawn, filtered and analyzed for β-Lap by UV spectrophotometry at the wavelength of 257.2 nm. An association constant (K_c) was calculated from the linear relationship between the dissolved β-Lap and the concentration of CD in the phase solubility diagram by the following equation based on the assumption that 1:1 complex was formed.

$$K_{1:1} = \frac{\text{slope}}{\text{intercept} \times (1-\text{slope})} \qquad (1)$$

Fluorescence study of β-CD-β-Lap inclusion complex- In order to obtain the correct excitation and emission wavelength, the emission spectra of β-Lap were preformed by LS45 Luminescence Spectrometer from Perkin Elmer instruments with 100 nm/min for the scan speed and 10 nm for both excitation slit width and emission slit width.

Fluorescence study of complex- A stock solution of β-Lap 4 x 10^{-6} mol/l in PBS was prepared. Different amounts of β-CD were dissolved in this β-Lap stock solution to get 1.54 x 10^{-4} mol/l to 1.58 x 10^{-2} mol/l of β-CD. The solution was vigorously stirred at 25 °C overnight until equilibrium before measurement. Every spectrum used β-CD blank for background subtraction.

Cytotoxicity assays- The cytoxicity of β-CD-β-Lap inclusion complex was studied by DNA assays as previously described (Planchon et. al., Exp. Cell Res., 2001; Pink et al., JBC, 2000; Tagliarino et. al., JBC, 2001). Briefly, 5.0 x 10^{x} cells/ml of MCF-7:WS8 cells were seeded in the 48-well plates **(Fisher)** and then incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were wash 2 times with PBS, and incubated for 4 h in an humidified atmosphere (5% CO_2 -90% air, 37 °C) with DMEM culture medium containing different concentrations of β-CD-β-Lap inclusion complexes, ranging from 0-20 μM. In order to study the effect of pure β-CD on cell growth, control experiments with the same amount (percentage) of β-CD in the 0 to 20 μM range were performed. The same experimental setup was prepared by using β-Lap dissolved in DMSO at the same concentrations as β-Lap-β-CD complexes. After 4h drug exposures, cells were washed three times and allowed to grow for 10 days, or cell growth was interrupted at various times to monitor growth inhibition. Treated or untreated cells were then lysed by alkaline hydrolysis and DNA content per lane read as described (Planchon et. al., Exp. Cell Res., 2001; Pink et al., JBC, 2000; Tagliarino et. al., JBC, 2001). Results and Discussion:

Dissolution studies - The results showed that the amount of β-Lap dissolved from β-CD containing solution was higher than β-Lap alone at each time point. It took at least 24 h to reach equilibrium.

Solubility study- The effect of cyclodextrins on the aqueous solubility of β-Lap was then evaluated by the phase solubility method. The phase solubility diagrams of β-Lap with four CDs in water are shown in Fig. 2. Only ß-cyclodextrin demonstrated significant solubility of ß-lapachone (Fig. 2).

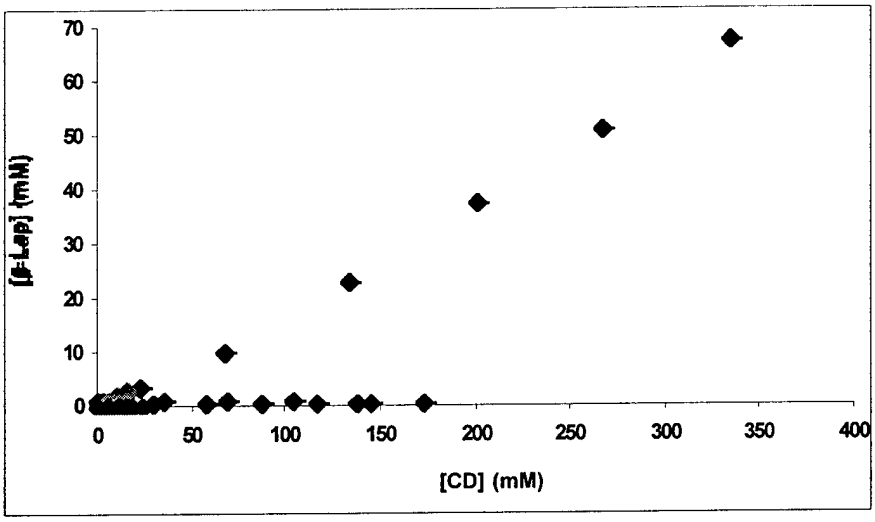


Figure 2. Solubility of β-Lap as a function of β-CD concentration at 25 °C

The solubility of β-Lap increased in a linear fashion as a function of alpha, beta and hydroxypropyl-beta-cyclodextrin concentration, and the resulting solubility curve was classified as type A_L. The solubility plot for the β-CD has a higher slope than that of α-CD, but the solubility of β-Lap was limited by the solubility of β-CD (18.2 mg/ml).

The solubility limit of β-CD could, however, be solved by using HPβ-CD, which has very high water solubility (> 500 mg/ml) than ß-CD. The solubility plot has slightly different slope from that of β-CD. On the other hand, gamma-cyclodextrin showed a typical B_S-type solubility curve. It was found that increasing the β-CD molar concentration linearly enhanced β-Lap solubility, and could also be classified as type A_L. This was because β-Lap exhibited very low solubility in water, but enters the hydrophobic cavity of β-CD as depicted in Fig. 1. In the other words, as expected these data strongly indicate the formation of inclusion complexes between β-CD and β-Lap. The solubility of β-Lap increased about ten-fold (from 0.16×10^{-3} to 2.80×10^{-3} mol/l) in solution containing 0.0158 mol/l β-CD, which is the maximum solubility of β-CD. The association constant calculated from equation 1 assuming the formation of a complex with a 1:1 complex is $1.28 \pm 0.3 \times 10^3$ M^{-1}. However, the addition of β-CD did not significant change the absorption spectra of β-Lap.

Fluorescence study. The emission spectrum of β-Lap is shown in Fig. 3. The emission bands were constant at 436 nm when the excitation wavelengths were changed from 257 to 360 nm. It was also found that the excitation wavelength at 330 nm gives the highest emission intensity.

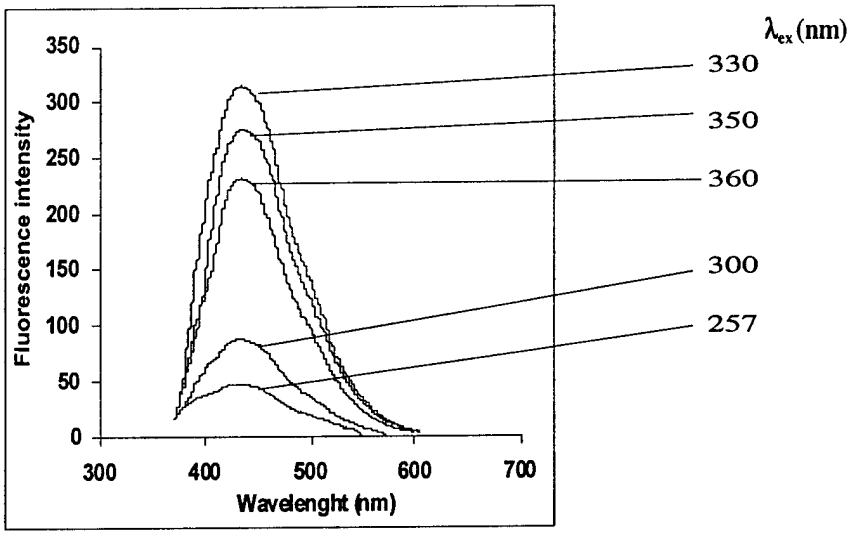


Figure 3. Emission spectra of β-Lap at various excitation wavelengths.

The effect of the concentration of β-CD on the fluorescence spectra obtained by exciting at 330 nm is shown in Fig. 4. ß-CD uniquely quenched the emission intensity, and a blue shift in the emission maximum spectrum was found to be around 6 nm. The blue shift indicates β-Lap experience a less polar environment causing by the hydrophobic cavity of β-CD.

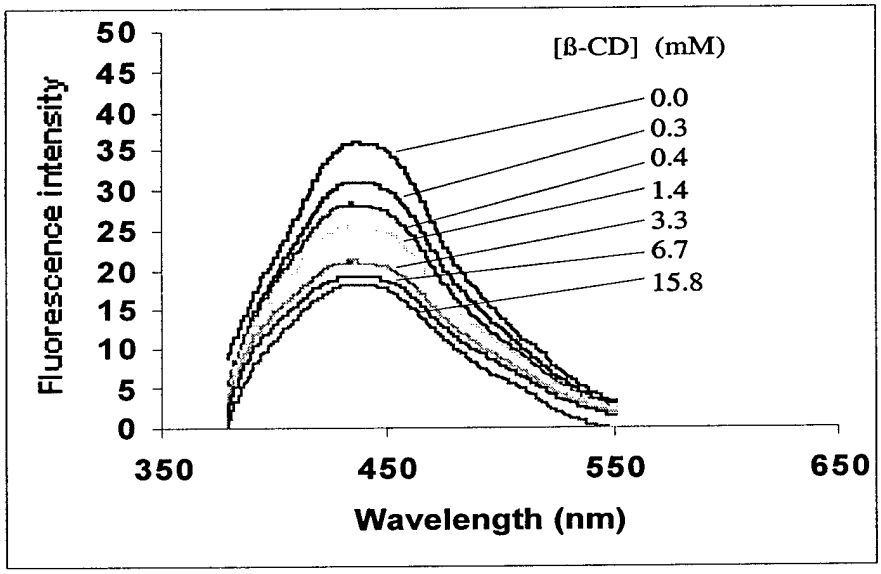


Figure 4. Emission spectra of β-Lap (4 x 10^{-6} mol/l) in various β-CD concentrations at 25 °C.

The plot shows that the emission intensity of ß-lapachone decreased with increasing β-CD concentrations. The change of fluorescence intensity of β-Lap by the addition of β-CD was another indication of the formation of the inclusion complex between β-CD and β-Lap. Considering that there are two fluorescence substances in the system, the free drug (S) and the complexed drug (SL) having

lower fluorescence intensity than the free drug, the total fluorescence intensity, which decreases as β-CD (L) increases and then reaching a constant value, is the sum of the contribution of these two substances. In this kind of system, the molar ratio (R) of free drug and complexed drug at each β-CD concentration can be demonstrated by the equations 2 and 3, respectively.

$$R_S = (F_o - F_T) - (F_o - F_i) \ / (F_o - F_T) \quad (2)$$

$$R_{SL} = (F_o - F_i) \ / (F_o - F_T) \quad (3)$$

where F_o and F_T denote the fluorescence intensity of β-Lap in the absence and in the presence of excess β-CD, F_i stands for the total fluorescence intensity of the β-Lap in β-CD

Assuming the formation of the 1:1 β-CD-β-Lap inclusion complex, the concentration of S and SL in the association constant as shown in fig. 1 can be replaced by R_S and R_{SL}, respectively resulting a scatchard equation (2) as shown below.

$$R_{SL} / [\beta\text{-CD}] = -R_{SL} K_{1:1} + K_{1:1} \quad (2)$$

Rearrangement of equation 2 provides equation 3, which can be used to represent and determine the association constant.

$$(F_o - F_i) / [\beta\text{-CD}] = -(F_o - F_i)K_{1:1} + (F_o - F_T)K_{1:1} \quad (3)$$

The plot between $(F_o\text{-}F_i) / [\beta\text{-CD}]$ versus $(F_o\text{-}F_i)$ shown in Fig. 5 exhibit a linear relationship for the whole range of β-CD concentration indicating the agreement with 1:1 complex assumption in derivation of equation 2. A $K_{1:1}$ value of $1.10 \pm 0.09 \times 10^3$ M^{-1} is evaluated from the ratio of intercept/slope, which closes to a $K_{1:1}$ value calculated from the phase solubility method ($1.28 \pm 0.03 \times 10^3$ M^{-1}).

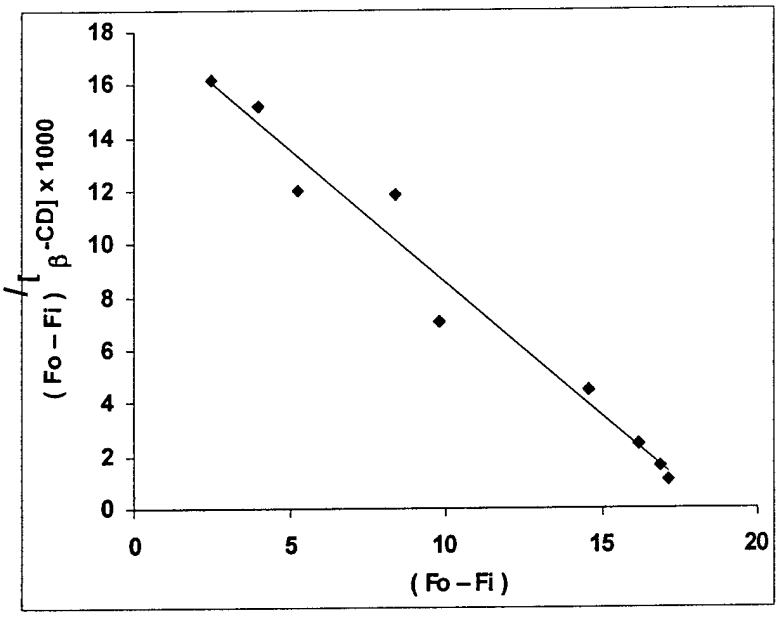


Figure 5. Plot of (F_o-F_i) / [β-CD] versus (F_o-F_i) obtained from β-Lap (4×10^{-6} mol/l) in various β-CD concentrations at 25 °C.

DNA assay

The growth inhibition of NQ01-expressing MCF-7 cells exposed to different concentrations of β-CD-β-Lap inclusion complexes, β-Lap dissolved in DMSO, and pure β-CD are shown in Fig. 6.

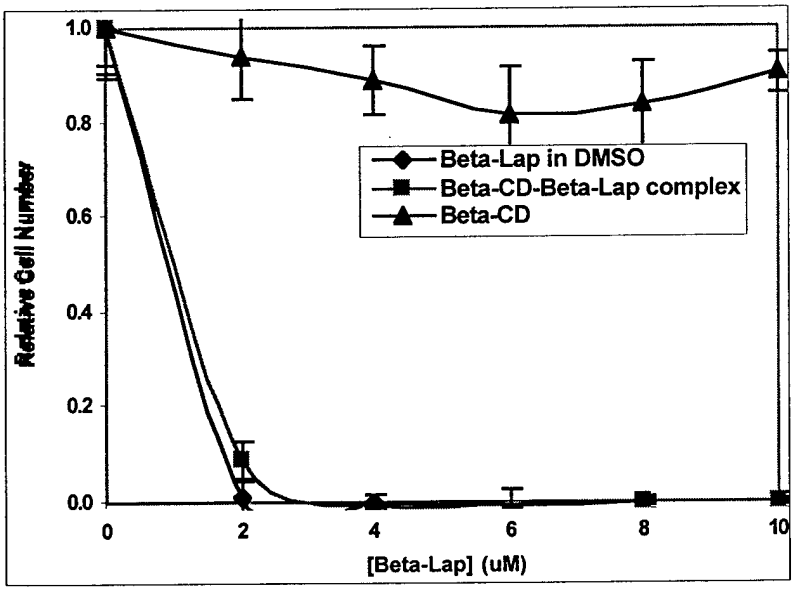


Figure 6. Cells viability in the presence of β-CD, β-CD-β-Lap inclusion complex and β-Lap/DMSO

The results clearly demonstrated that ß-CD-ß-lap inclusion complexes were not significantly different from the cytotoxicity caused by ß-lap dissolved in DMSO. Cytotoxicity as measured in these growth inhibition assays was previously demonstrated to be caused by cell death (apoptosis) and correlated well with lethality as measured by clonogenic lethality assays (Pink et. al., JBC, 2000). Control experiments using pure β-CD up to 20% by volume (similar to than used for the 20 μM ß-lap dose range, showed no cytotoxic effects. These data indicate that the activity of β-CD-β-Lap inclusion complex and β-Lap dissolved in DMSO resulted in near identical cytotoxicity responses. These data also demonstrated the success of improvement of β-Lap solubility by β-CD and the formation of the inclusion complex between β-CD and β-Lap. These ß-CD-ß-lap complex inclusions were then used to inject BDF1 mice to evaluate the toxicity of ß-lapachone in mice. Such studies are required prior to exploring the efficacy of ß-lapachone in mice against human prostate LNCaP xenografts grown in male nude mice.

Conclusions: In this first year of this grant, ß-lapachone (β-lap) was successfully formed in 1:1 inclusion complexes with β-CD, resulting in a significant enhancement of β-lap solubility. Phase solubility and fluorescence spectroscopy showed the important data for calculating the association constant of the 1:1 β-Lap-β-CD inclusion complex. The biological activity of β-CD-β-Lap inclusion complex and β-Lap dissolved in DMSO studied by DNA assay showed the same response. Further investigation demonstrated that an analogue of ß-CD, HP-ß-CD, was a more effective

ß-Lapachone (ß-lap) Toxicity studies: These studies have only recently begun. In our initial investigation, the following results were obtained. Female C57Blk/6 mice (4 per group) were injected with 5-150 mg/kg once every other day for 10 injections total. Table 1 illustrates the results:

Table 1. HP-ß-CD significantly enhances the bioavailability of ß-lapachone (ß-lap).

ß-Lap Dose	Weight Loss	Death
0 (HP-ß-CD Alone)	Weight Gain	0/4
5	Weight Gain	0/4
10	Weight Gain	0/4
20	Weight Gain	0/4
100	3 gms in 2 days	4/4
125	No Weight Loss	4/4 Immediate death, 1 h
150	No Weight Loss	4/4 Immediate death, 1 h

The results in Table 1 strongly indicate a significant increase in bioavailability of ß-lap due to solubility in HP-ß-CD, compared to previous reported optimal doses of ß-lapachone by Li and Pardee et al., PNAS, 1999. Thus, in the first year we have generated tetracycline-inducible LNCaP cells, explored and confirmed the targeting of NQ01 in LNCaP cells by ß-lapachone using constitutive-expressing cells (Planchon et. al., Exp. Cell Res., 2000).

Development of Tet^{ON}-inducible LNCaP cells. Tet-inducible LNCaP cells were developed as described in the original grant proposal. Briefly, the tet-repressor was transfected into LNCaP cells and stable clones (4 of 20) were isolated that induced TRE-luciferase greater than 40-fold after exposure to doxycycline (10 μg/ml), yet had low basal level expression of luciferase and were therefore considered less leaky than other clones isolated. We have recently constructed a TRE-NQ01 mammalian expression vector for subsequent transfection into the Tet^{ON}-expressing LNCaP cells.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

We have accomplished the following objectives of this grant. We have determined/generated that:

Aim #1:

1. NQ01-expressing LNCaP cells are sensitive to ß-lapachone (ß-lap).
2. As expected, Dicoumarol suppressed ß-lap-induced cell death (apoptosis).
3. LNCaP cells are able to conditionally induce a gene of interest in response to exogenously administered tetracycline

Aim #2:

4. LNCaP cells are able to conditionally induce a gene of interest in response to exogenously administered tetracycline.
5. A TRE-NQ01 mammalian expression vector has been made and confirmed by DNA sequencing.
6. The solubility of ß-lapachone can be significantly increased by the use of ß-CD or HP-ß-CD.
7. The use of ß-CD or HP-ß-CD significantly enhances the bioavailibility of ß-lapachone, now allowing xenograft studies to be easily performed and Aim #2 completed.
8. Several additional potential routes of drug delivery have been developed, including millirods and microspheres. The drug is trapped in biodegradable, biopolymers with glucose and drug release can be controlled. The millirods will be used for brachytherapy, the microspheres for systemic or aerosol drug delivery.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes to include:

Prior Appendix Items Already Supplied to the DOD In Past Reports:

None

CURRENT LIST OF PUBLICATIONS RESULTING FROM THIS AWARD
PAPERS PUBLISHED IN PEER-REVIEWED JOURNALS (Enclosed):

Tagliarino, C., Pink, J.J., Dubyak, G.R., Nieminen, A-L., and Boothman, D.A. Calcium is a key signaling moleculae in ß-lapachone-mediated cell death. 2001; J. Biol. Chem. 276(22): 19150-19159.

Planchon, S.M., Pink, J.J., Tagliarino,, C., Bornmann, W.G., Varnes, M.E., and Boothman, D.A. ß-Lapachone-induced apoptosis in human prostate cancer cells: involvement of NQO1/xip3. 2001; Exp. Cell Res., 267: 95-106.

Tagliarino, C. Pink, J.J. and Boothman, D.A. Calpains and apoptosis. 2001; Korean J. Biol Sci., 5: 267-274.

PAPERS PUBLISHED IN NON-PEER-REVIEWED JOURNALS (Not Enclosed).

Abstracts and Presentations Related to this Grant:

None.

Patents and licenses applied for and/or issued.

We are currently writing up a patent on the use of ß-cyclodextrin, as well as microspheres and millirods for the delivery of ß-lapachone *in vivo*. Specifically, we are excited about the possibility of combining millirod delivery of ß-lapachone with radioactive seeds used for brachytherapy.

Degrees Obtained During This Award.

1. Tagliarino, Colleen Ph.D., Case Western Reserve University, Dept. Pharmacology, October, 2001.

Development of Cell Lines, Tissue or Serum Repositories:

-LNCaP NQ CLONES 1-10: human NQ01-deficient cells stably transfected with CMV-directed NQ01.
-vector-alone LNCaP cells.
-Tetracycline-inducible, LNCaP cells.

Informatics such as databases and animal models, etc. None

Funding applied for based on work supported by this award.

None.

Employment or research opportunities applied for and/or received on experiences/training supported by this award.

Colleen Tagliarino, Ph.D. Post-doctoral Fellow, Johnson and Johnson Drug Development Center, Philadelphia, PA.

CONCLUSIONS: Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the annual and final reports.

The main progress on this grant has been the development of novel routes of drug administration. ß-Cyclodextrin derivatives have been used to suspend ß-lapachone and greatly increase its bioavailability, while not altering its cytotoxic or apoptotic antitumor potential, nor its mechanism of action in the cell. Animal studies have begun and we have already seen a dramatic increase in bioavailability of the drug in mice. Tetracycline (doxycycline)-inducible LNCaP cells have been developed and we have constructed a TRE-NQ01 mammalian expression vector for tet-conditional expression of NQ01.

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PAPERS PUBLISHED IN PEER-REVIEWED JOURNALS (Enclosed):

Planchon, S.M., Pink, J.J., Tagliarino,, C., Bornmann, W.G., Varnes, M.E., and Boothman, D.A. ß-Lapachone-induced apoptosis in human prostate cancer cells: involvement of NQO1/xip3. Exp. Cell Res., 267: 95-106, 2001.

PAPERS PUBLISHED IN NON-PEER-REVIEWED JOURNALS (Not Enclosed).

Tagliarino, C. Pink, J.J. and Boothman, D.A. Calpains and apoptosis. Korean J. Biol Sci., 5: 267-274, 2001.

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Appendix

Grant # DAMD17-01-0038

"Exploiting IR-inducible NQ01 levels using ß-lapachone, a novel apoptotic agent"

I Published papers as a result of this grant

1. **Planchon, S.M., Pink, J.J., Tagliarino,, C., Bornmann, W.G., Varnes, M.E., and Boothman, D.A. ß-Lapachone-induced apoptosis in human prostate cancer cells: involvement of NQO1/xip3. 2001; Exp. Cell Res., 267: 95-106.**

2. **Tagliarino, C., Pink, J.J., Dubyak, G.R., Nieminen, A-L., and Boothman, D.A. Calcium is a key signaling moleculae in ß-lapachone-mediated cell death. 2001; J. Biol. Chem. 276(22): 19150-19159.**

3. **Tagliarino, C. Pink, J.J. and Boothman, D.A. Calpains and apoptosis. 2001; Korean J. Biol Sci., 5: 267-274.**

II. Papers in Preparation:

1. **Nasongkla, N., Wiedmann, A.F., Boothman, D.A. and Gao, J. Enhancement of solubility and biological activity of ß-lapachone by forming inclusion complexes with cyclodextrins. In Preparation, 2002.**

Experimental Cell Research **267,** 95–106 (2001)
doi:10.1006/excr.2001.5234, available online at http://www.idealibrary.com on IDEAL®

β-Lapachone-Induced Apoptosis in Human Prostate Cancer Cells: Involvement of NQO1/xip3

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β-Lapachone (β-lap) induces apoptosis in various cancer cells, and its intracellular target has recently been elucidated in breast cancer cells. Here we show that NAD(P)H:quinone oxidoreductase (NQO1/xip3) expression in human prostate cancer cells is a key determinant for apoptosis and lethality after β-lap exposures. β-Lap-treated, NQO1-deficient LNCaP cells were significantly more resistant to apoptosis than NQO1-expressing DU-145 or PC-3 cells after drug exposures. Formation of an atypical 60-kDa PARP cleavage fragment in DU-145 or PC-3 cells was observed after 10 μM β-lap treatment and correlated with apoptosis. In contrast, LNCaP cells required 25 μM β-lap to induce similar responses. Atypical PARP cleavage in β-lap-treated cells was not affected by 100 μM zVAD-fmk; however, coadministration of dicoumarol, a specific inhibitor of NQO1, reduced β-lap-mediated cytotoxicity, apoptosis, and atypical PARP cleavage in NQO1-expressing cells. Dicoumarol did not affect the more β-lap-resistant LNCaP cells. Stable transfection of LNCaP cells with NQO1 increased their sensitivity to β-lap, enhancing apoptosis compared to parental LNCaP cells or vector-alone transfectants. Dicoumarol increased survival of β-lap-treated NQO1-expressing LNCaP transfectants. NQO1 activity, therefore, is a key determinant of β-lap-mediated apoptosis and cytotoxicity in prostate cancer cells. © 2001 Academic Press

Key Words: **β-lapachone; apoptosis; NQO1; X-ray-inducible protein 3 (xip3); prostate cancer; atypical PARP cleavage; p53 cleavage.**

INTRODUCTION

β-Lapachone (β-lap, 3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione)[2] is a naturally occurring *o*-naphthoquinone present in the bark of the Lapacho tree (*Tabebuia avellanedae*) native to South America. The purified drug has anti-trypanosomal, -fungal, -tumor, and -HIV properties and induces apoptosis in a variety of cell types [1]. The mechanism of action and intracellular target(s) of the compound have, however, remained elusive and prevented the preclinical development of this drug for use as an antitumor or antiviral agent. Using a series of *in vitro* assays, proposed mechanisms of action for this drug have included: (a) activation of topoisomerase (Topo) I [2]; (b) induction of apoptosis [3]; (c) inhibition of Topo I [1, 4, 5]; (d) inhibition of Topo II-α [6]; and (e) suppression of NF-κB activation [7]. β-lap can induce apoptosis in several cell systems, including leukemic (HL-60), prostate, and breast cancer cell lines [1, 3, 5]. The apoptotic response caused by β-lap was independent of both p53 status and androgen dependence in human prostate cancer cell lines [1]. Camptothecin (CPT), a Topo I poison, induces classical caspase-mediated apoptotic responses [3].

We recently showed that the enzymatic activity of NQO1 in breast cancer cell lines is a key determinant for β-lap-mediated cytotoxicity [8]. NAD(P)H:quinone reductase (NQO1, DT diaphorase, xip3, EC 1.6.99.2) is a flavoenzyme that catalyzes the two-electron reduction of quinones into their hydroquinone form, bypassing the often mutagenic semiquinone intermediate and the formation of free radicals [9, 10]. NQO1 detoxifies many quinones [e.g., menadione] [11, 12] and bioactivates other compounds, such as mitomycin C, streptonigrin, or E09 [13–16]. NQO1 gene expression is widespread, with detectable levels in human heart, brain, placenta, lung, skeletal muscle, kidney, and pancreatic tissue, and low or absent in human liver [17]. More

[1] To whom correspondence and reprint requests should be addressed. Fax: (216) 368-1142. E-mail: dab30@po.cwru.edu.

[2] Abbreviations used: β-lap, β-lapachone; b5R, NADH:cytochrome b5 reductase; CFA, colony-forming ability; CPT, camptothecin; NQO1, NAD(P)H:quinone oxidoreductase 1; PARP, poly(ADP-ribosyl) polymerase; P450r, NADH:cytochrome P-450 reductase; SD, standard deviation; TUNEL, terminal dUTP nick-end labeling; zVAD-fmk, benzyloxycarbonyl-val-ala-asp (OMe) fluoromethylketone.

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importantly, NQO1 levels have been shown to be significantly up-regulated (5- to 20-fold above adjacent normal tissue) in several forms of cancer, including breast and non-small-cell lung tumors [17–19]. Such elevations in certain cancers make NQO1 a potential target for the development of tumor-directed therapies involving β-lap or its derivatives [8, 20].

Apoptosis is a genetically programmed form of cell death, the initiation and execution of which are thought to be the basis of lethality caused by many chemotherapeutic agents [21–23]. Cells undergoing apoptosis exhibit characteristic changes, including cell shrinkage, membrane blebbing, chromatin condensation, internucleosomal DNA cleavage, and cleavage of specific intracellular substrates involved in cell structure, DNA repair [e.g., poly(ADP-ribosyl) polymerase (PARP)], and general homeostasis. These intracellular alterations are often the result of activation of a family of apoptotic proteases, including caspases [24–28] and/or calpains [29–32].

Caspases are activated by multiple signaling pathways during apoptosis and typically result in the cleavage of PARP; the full-length 113-kDa polypeptide is cleaved to diagnostic 89-kDa and 24-kDa proteins [33]. In HL-60 cells, CPT induced an apoptotic pathway that included activation of the caspases, leading to classical PARP cleavage [20, 34]. HL-60 cells treated with β-lap also activated the caspases [20]. However, a different cell death response appeared to be stimulated by the drug in various human breast cancer cells [3, 35]. In many breast cancer (especially MCF-7:WS8) cells, an atypical PARP fragmentation *in vivo* was noted at times and doses correlating with apoptosis. Apoptotic responses induced by β-lap were monitored by DNA fragmentation (cells staining positive in a TUNEL assay), dephosphorylation of pRb, lamin B cleavage, cleavage of p53, and an atypical cleavage of PARP, leading to an ~60-kDa fragment. β-Lap-mediated apoptosis was thought to involve the activation of a calpain-like protease due to the specific calcium-dependent cleavage of both p53 and PARP [8]. Recently, our laboratory discovered that NQO1 was the key determinant of β-lap cytotoxicity in human breast cancer cell lines [8].

In this report, we demonstrate that NQO1 is also a key determinant of β-lap-induced apoptosis and lethality in human prostate cancer cell lines, suggesting a general mechanism of activation for the compound. Variations in NQO1 activity dramatically affected the sensitivity of human prostate cancer cell lines to β-lap, as determined by comparing various cell lines expressing different levels of the enzyme, by transfection of NQO1 expression vectors into enzyme-deficient cells, and/or by the use of the NQO1 inhibitor, dicoumarol. Coadministration of dicoumarol abrogated β-lap-mediated cytotoxicity and downstream apoptotic end points in NQO1-expressing, but not in NQO1-deficient, human prostate cancer cell lines. Transfection of NQO1-deficient LNCaP cells with NQO1 significantly enhanced sensitivity (apoptosis, substrate proteolysis, and lethality) to β-lap, which was subsequently blocked by dicoumarol coadministration.

MATERIALS AND METHODS

Compounds and drug preparations. β-Lap was synthesized and dissolved in DMSO as described [1]. CPT and dicoumarol were obtained from Sigma Chemical Co. (St. Louis, MO) and prepared in DMSO or water, respectively [3]. zVAD-fmk and zDEVD-fmk were obtained from Enzyme Systems Products (Dublin, CA). Control treatments containing an equivalent percentage of DMSO were included as described [1–3]. The highest DMSO concentration used was 0.2%, which did not affect survival, cell growth, or apoptosis in various human breast or prostate cancer cells examined [1–3].

Cell culture conditions. PC-3, DU-145, and LNCaP human prostate cancer cells were obtained from Dr. George Wilding (University of Wisconsin–Madison) and grown in Dulbecco's minimal essential medium (DMEM) with 5% FBS at 37°C in a humidified 5% CO_2–95% air atmosphere as described [36–38]. Tests for mycoplasma infection, using the Gen-Probe Rapid Detection Kit (Fisher Scientific, Pittsburgh, PA), were performed quarterly and all cell lines were negative.

Drug treatments. For all experiments, cells were plated, allowed at least 24 h to initiate log-phase growth, and then exposed to β-lap or CPT at indicated doses for 4 h. After exposure, drug-containing media were removed and replaced with complete media. Dicoumarol was administered (50 μM) concomitantly with β-lap or CPT for 4 h as described above. For zVAD-fmk exposures, cells were pretreated with 100 μM zVAD-fmk for 30 min or treated with media alone. β-Lap or CPT was then coadministered in the presence or absence of zVAD-fmk for 4 h. All drug-containing media were then removed and replaced with media containing 100 μM zVAD-fmk alone or with fresh, non-drug-containing media.

Stable transfection of LNCaP cells with NQO1. Log-phase LNCaP cells were seeded onto 6-well dishes at 2×10^5 cells/well and allowed to attach overnight. The following day, 1.0 μg of BE8 plasmid DNA, containing human NQO1 cDNA under the control of the CMV promoter in the pcDNA3 constitutive expression vector [39], was added into each of three wells using standard calcium phosphate transfection methodology [40]. After 2 days of growth without selection, cells were exposed to 350 μg/ml geneticin (G418, GIBCO BRL, Gaithersburg, MD). A stable, pooled population was established after approximately 3 weeks of growth in media containing 350 μg/ml G418. Clonal transfectants were finally derived from the pooled population by limiting dilution cloning. Isolated transfectants were then analyzed for NQO1 expression and enzymatic activity as described below and under Results (Table 1).

Colony-forming ability assays. Anchorage-dependent colony-forming ability (CFA) assays were performed [1, 41]. For CFA assays, cells were seeded at 1–2000 viable cells per dish in 35 mm^2-tissue culture plates (with grids) and incubated overnight. Plated cells were then treated with equal volumes of media containing β-lap at various concentrations for 4 h. Control cells were treated with DMSO equivalent to the highest dose of β-lap used. β-Lap exposures in the presence or absence of 50 μM dicoumarol were performed as indicated above and under Results. Colonies were allowed to grow for 10–14 days, with one change of medium at day seven. Plates were stained with 1.0% crystal violet in 20% EtOH and destained with water, and colonies of >50 normal-appearing cells were counted [1, 41].

TUNEL assays. Flow cytometric TUNEL analyses were performed to measure DNA fragmentation, sub-G_0/G_1 cell populations, and changes in cell cycle distribution following various drug treatments [1, 35]. TUNEL assays were performed using APO-DIRECT as described by the manufacturer (Pharmingen, San Diego, CA). Samples were analyzed in an EPICS Elite ESP flow cytometer using an air-cooled argon laser at 488 nm, 15 mW (Beckman Coulter Electronics, Miami, FL). Propidium iodide was read with a 640-nm long-pass optical filter. FITC was read with a 525-nm band-pass filter. TUNEL analyses were performed using the Elite acquisition software provided with the instrument. Sub-G_0/G_1 data were analyzed using ModFit (Verity Software House, Inc., Topsham, ME) [1, 3, 35]. Results presented are means ± SD for at least three separate experiments, repeated in duplicate.

Western immunoblot analyses. Control or treated human prostate cancer cells were examined for changes in PARP, p53, and lamin B and for levels of NQO1. Actin was used as a loading control. Briefly, control or treated cells were washed in ice-cold PBS and lysed in loading buffer [62.5 mM Tris, pH 6.8, 6M urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, 5% 2-mercaptoethanol (freshly added)]. Samples were sonicated with a Fisher Scientific Sonic Dismembrator (model 550) fitted with a microtip probe and stored at −20°C for later analyses as described [35]. Equivalent amounts of protein were incubated at 65°C for 15 min and polypeptides were separated by SDS–PAGE. Separated proteins were then transferred to immobilon-P membranes (Millipore, Danvers, MA), and equivalent protein loading was confirmed by Ponceau S staining [0.2% Ponceau S (w/v) in 3% trichloroacetic acid (w/v) and 3% sulfosalicylic acid (w/v)] (Sigma) using standard techniques. Western immunoblots were incubated with PBS containing 0.2% Tween 20 and 10% FBS for 1 h to prevent nonspecific binding. Membranes were then incubated overnight with primary antibodies diluted in the same blocking buffer at 4°C. Primary antibodies included separate, and sometimes in combination, exposures to anti-PARP C2-10 (Enzyme Systems Products), anti-p53 DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-lamin B (Calbiochem, San Diego, CA), and anti-actin (Amersham Pharmacia Biotech, Piscataway, NJ). An NQO1 antibody was contained in medium from a mouse hybridoma, clone A180, and used according to previously published procedures [42]. Membranes were washed in PBS containing 0.2% Tween and then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h. Western immunoblots were then washed in PBS containing 0.2% Tween, developed with enhanced chemiluminescence (ECL) substrate (Amersham, Arlington Heights, IL), and exposed to Fuji X-ray film. All Western immunoblots shown below are representative of experiments repeated at least three times.

Preparation of S9 supernatants. Cellular extracts for enzyme assays were prepared from cells in mid- to late-log-phase growth. Cells were harvested by trypsinization (0.25% trypsin and 1 mM EDTA), washed twice in ice-cold, phenol red-free Hank's balanced salt solution, and resuspended in a small volume of PBS, pH 7.2, containing 10 μg/μl aprotinin. Cell suspensions were sonicated four times on ice using 10-s pulses, then centrifuged at 14,000*g* for 20 min. S9 supernatants were aliquoted into microfuge tubes and stored at −80°C for later use as described below.

NQO1, cytochrome b5 reductase, and cytochrome P450 reductase enzyme assays. Three general reductase enzyme assays were completed as described [43–46]. Enzyme reactions contained 77 μM cytochrome *c* (practical grade, Sigma) and 0.14% bovine serum albumin in Tris–HCl buffer (50 mM, pH 7.5). NQO1 activity was measured using NADH (200 μM) as the immediate electron donor and menadione (10 μM) as the intermediate electron acceptor. Each assay was repeated in the presence of 10 μM dicoumarol, and the activity attributed to NQO1 was that inhibited by dicoumarol [44, 47]. NADH:cytochrome b5 reductase (b5R) was measured using NADH (200 μM) as the electron donor, and NADH:cytochrome P-450 reductase (P450r) was measured using NADPH (200 μM) as the electron donor [54] in a Beckman DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA). Reactions were performed at 37°C and were initiated by addition of S9 supernatants. Varying amounts (10 to 40 μl) of S9 supernatants were used to ensure linearity of enzyme rates with protein concentration. Enzyme activities were calculated as nmol cytochrome *c* reduced/min/mg protein, based on the initial rate of change in OD at 550 nm. An extinction coefficient of 21.1 mM/cm was used for cytochrome *c*.

TABLE 1

NQO1, Cytochrome b5R, and Cytochrome P450 Reductase Enzyme Activity Levels in Prostate Cancer Cell Lines

Cell line	NQO1	b5R	P450
LNCaP	9.0 ± 2.4	17 ± 3.2	7.0 ± 2.3
DU-145	500 ± 48	39 ± 3.3	6.8 ± 0.6
PC-3	740 ± 100	38 ± 5.1	7.9 ± 1.1
LN-pcDNA3 Clone 5	3.9 ± 0.5	22 ± 1.8	10 ± 2.8
LN-NQ Clone 1	140 ± 22	15 ± 1.9	9.5 ± 0.5
LN-NQ Clone 2	250 ± 61	34 ± 5.4	9.3 ± 4.5
LN-NQ Clone 3	290 ± 30	26 ± 1.5	9.8 ± 1.9
LN-NQ Clone 4	270 ± 30	22 ± 6.3	5.0 ± 1.0
LN-NQ Clone 10	210 ± 24	31 ± 3.3	9.7 ± 2.8

Note. Enzyme levels were measured in S9 supernatent whole cell lysates as described under Materials and Methods. Activities are reported as nmol cytochrome *c* reduced/min/mg protein.

RESULTS

Expression of NQO1 and p53 in human prostate cancer cells. In examining the apoptotic responses of human prostate cancer cells to β-lap, we noted that LNCaP was resistant to this drug. Since we previously published data that suggested NQO1 expression/activity was a critical determinant in the cytotoxicity of this drug [8], we examined LNCaP, PC-3, and DU-145 cells for expression of this two-electron reductase. DU-145 and PC-3 cells expressed NQO1 protein (Fig. 1) and demonstrated dicoumarol-sensitive enzyme activity (Table 1); enzymatic activity was measured by menadione-mediated, NQO1 reduction of cytochrome *c* as described under Materials and Methods [43]. In contrast, LNCaP cells did not express NQO1 protein or enzyme activity (Fig. 1 and Table 1).

Dicoumarol enhanced the survival of DU-145 or PC-3, but not LNCaP, cells following β-lap exposure. Since dicoumarol is a relatively specific inhibitor of NQO1, its effects on the survival of β-lap-treated prostate cancer cells were determined. Dicoumarol significantly enhanced the survival of β-lap-treated DU-145 or PC-3 cells (Fig. 2). The LD_{90} values for DU-145 and PC-3 cells were increased (i.e., the drug was less toxic) by three- and two-fold, respectively, compared to β-lap alone. For example, over 95% lethality was noted in DU-145 cells treated with 4.0 μM β-lap, whereas the same β-lap exposure was ineffective (>95% survival)

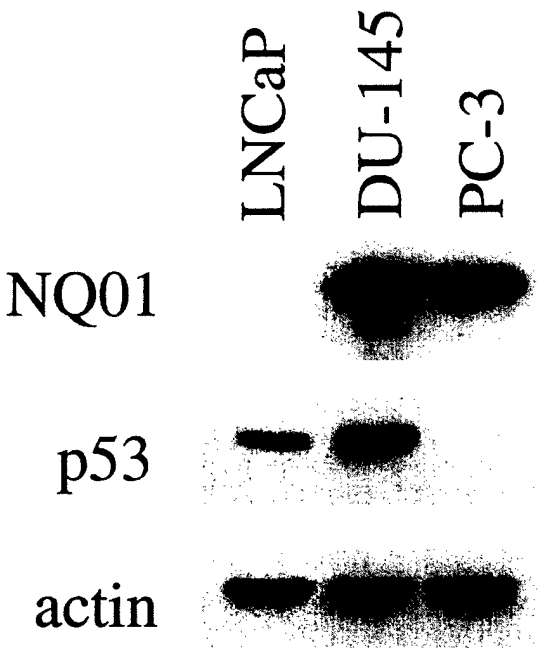

FIG. 1. NQO1 and p53 status of three human prostate cancer cell lines. Western immunoblot analyses of untreated lysates from three human prostate cancer cell lines, DU-145, PC-3, and LNCaP, were performed as described under Materials and Methods. Accurate loading was determined by monitoring actin levels.

when 50 μM dicoumarol was coadministered. In contrast, dicoumarol had no influence on the survival of β-lap-treated LNCaP cells, which also exhibited more intrinsic resistance to β-lap-mediated lethality (LD_{90}, 8.0 μM; LD_{99}, 12 μM) compared to DU-145 (LD_{90}, 3.5 μM; LD_{99}, 5.0 μM) or PC-3 (LD_{90}, 5.0 μM; LD_{99}, 7.5 μM) cells. β-Lap-treated LNCaP cells also exhibited threefold less apoptosis than either DU-145 or PC-3 cells when exposed to equitoxic concentrations [1]. In contrast, dicoumarol coadministration did not significantly affect the survival of LNCaP, DU-145, or PC-3 cells following CPT exposures (Fig. 2).

Dicoumarol blocked morphologic changes and apoptosis of DU-145 cells after β-lap treatment. In human breast cancer cells, β-lap induced morphologic changes indicative of apoptosis [3]. Similar alterations in morphology, such as chromatin condensation, cell shrinkage, and detachment occurred in DU-145 or PC-3 cells following 4-h β-lap exposures (shown are DU-145 cells, Fig. 3A). Addition of 50 μM dicoumarol significantly blocked β-lap-induced morphologic changes (Fig. 3A), and cells grew normally, consistent with enhanced survival as measured using CFA assays (Fig. 2).

We previously demonstrated the formation of an apoptotic sub-G_0/G_1 peak, representing apoptotic cells

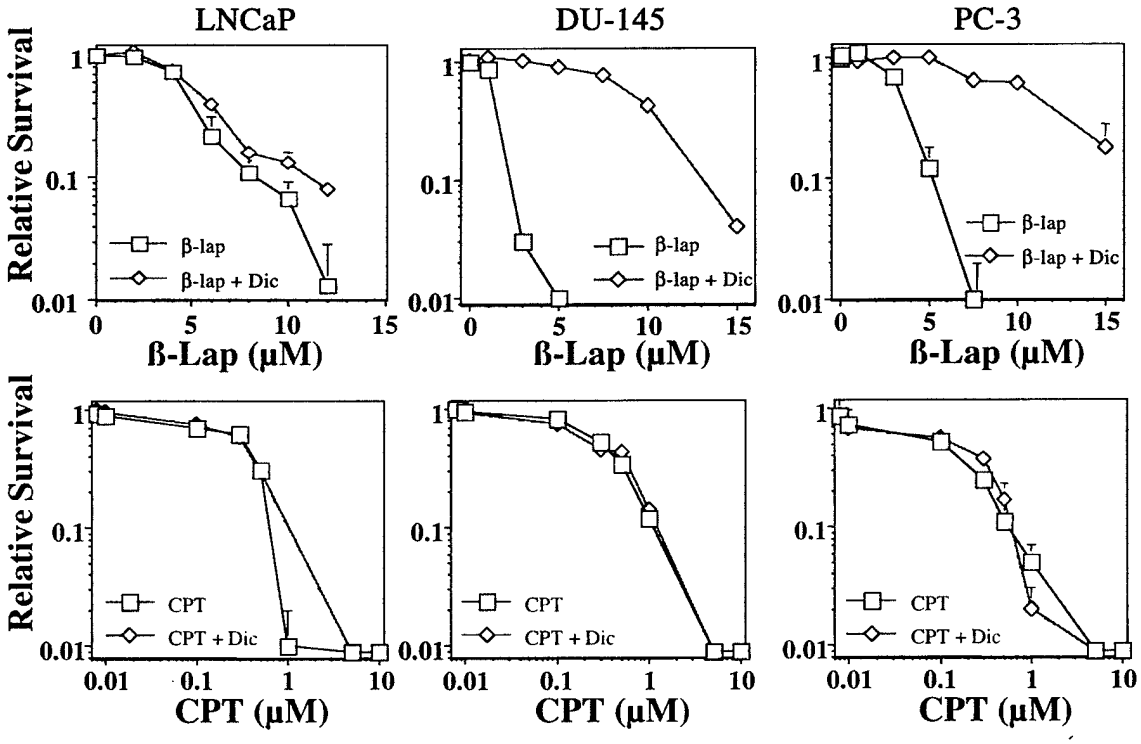

FIG. 2. Dicoumarol protects DU-145 and PC-3, but not LNCaP, human prostate cancer epithelial cell lines from β-lap-induced cytotoxicity. The survival of DU-145, PC-3, and LNCaP human prostate cancer cell lines following β-lap treatment, with or without dicoumarol coadministration, was determined by colony-forming-ability assays as described under Materials and Methods. β-Lap or CPT, with or without 50 μM dicoumarol cotreatments, was given as 4-h pulse treatments as described under Materials and Methods. Shown are the results (means $\pm$ SD) of three experiments repeated in duplicate.

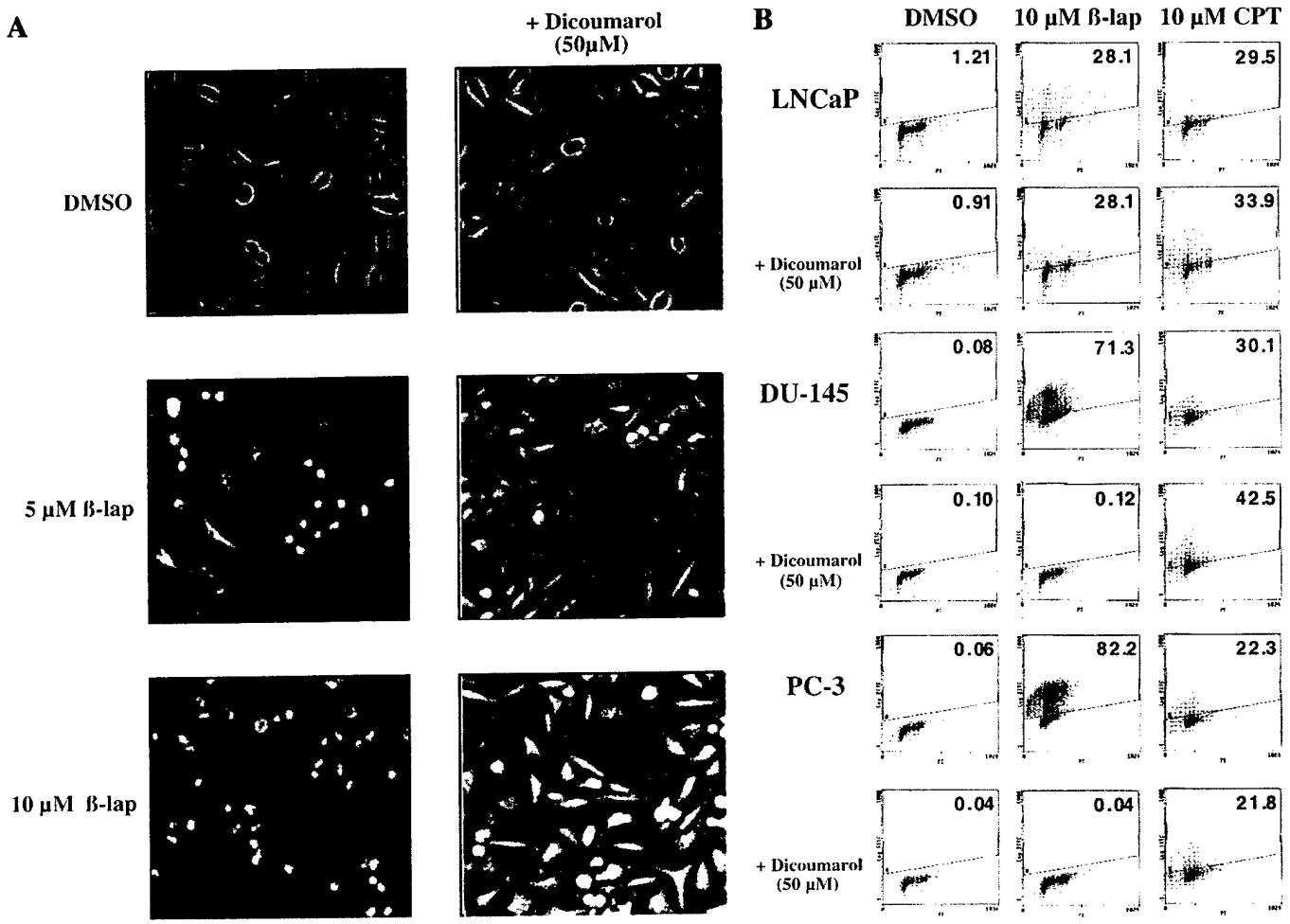

FIG. 3. (A) Dicoumarol blocks morphologic changes in DU-145 cells after β-lap treatment. DU-145 cells were treated with 5 μM or 10 μM β-lap, with or without 50 μM dicoumarol, for 4 h. At 24 h posttreatment, phase-contrast photomicrographs were taken of treated or control cells. Shown are representative photos of experiments repeated three or more times. Magnification, 100×. (B) Dicoumarol prevents apoptosis induced in human prostate cancer cells following β-lap, but not CPT. TUNEL assays to monitor apoptosis in β-lap- or CPT-treated human prostate cancer cells, with or without 50 μM dicoumarol coadministration, were performed 48 h following 4-h drug treatments. The percentage of cells that stained positive by TUNEL assay appears in the top right corner of each panel.

with fractional DNA content, in human prostate or breast cancer cell lines following β-lap treatment. DU-145 or PC-3 cells showed a prominent sub-G_0/G_1 population of cells. In contrast, NQO1-deficient LNCaP cells showed significantly lower levels of sub-G_0/G_1 cells [1]. To further characterize cell death responses in human prostate cancer cell lines after exposure to β-lap or CPT, TUNEL assays were performed to monitor apoptotic-related DNA fragmentation, with or without dicoumarol (Fig. 3B). DU-145 or PC-3 cells were positively stained by TUNEL (71.3 and 82.2%, respectively) after β-lap treatment, and these responses were abrogated by dicoumarol cotreatments. In contrast, β-lap-treated LNCaP cells exhibited a much lower percentage of apoptotic cells (28.1%), consistent with prior data [1]. Coadministration of dicoumarol did not affect β-lap-mediated responses in these cells. Treatment of each cell line with CPT resulted in only modest apoptosis (i.e., 22–43% apoptotic cells), as previously described [3]. Predictably, CPT-induced apoptosis was not affected by dicoumarol cotreatments (Fig. 3B).

Apoptotic substrate cleavage events in human prostate cancer cells after β-lap exposure. Human prostate cancer cell lines treated with β-lap exhibited the formation of an atypical ~60-kDa PARP polypeptide (Fig. 4A, open arrow), in contrast to classical, CPT-induced, caspase-mediated, 89-kDa PARP cleavage (Fig. 4A, closed arrow). Atypical 60-kDa PARP fragmentation was apparent in DU-145 and PC-3 cells treated with 10 μM β-lap and correlated well with apoptosis (Fig. 3B) [8, 35]. Furthermore, formation of β-lap-induced PARP cleavage was completely blocked by coadministration

of 50 μM dicoumarol (Fig. 4A), consistent with this NQO1 inhibitor's ability to prevent β-lap-mediated apoptosis (Fig. 3B) and lethality (Fig. 2). β-Lap-resistant LNCaP cells required a greater concentration of β-lap (25 μM) to induce an identical atypical PARP cleavage fragment. As with survival responses, coadministration of dicoumarol with β-lap did not affect the formation of the 60-kDa PARP cleavage fragment in β-lap-treated LNCaP cells (Fig. 4A). In contrast, all three human prostate cancer cell lines exhibited the formation of an 89-kDa PARP cleavage fragment (Fig. 4A, closed arrow) after 10 μM CPT exposures, correlating with the level of apoptosis observed (Fig. 3B). Dicoumarol coadministration had no effect on classical, caspase-mediated PARP cleavage after CPT exposures.

Global caspase inhibitors, such as zVAD-fmk, can inhibit the activation of many of the caspases and their downstream events (i.e., substrate proteolysis) [5, 26]. Addition of 100 μM zVAD-fmk completely abrogated the formation of CPT-induced PARP cleavage (89 kDa), (Fig. 4B). In contrast, atypical PARP cleavage noted in β-lap-treated DU-145 cells (open arrow) was not affected by 100 μM zVAD-fmk, suggesting either that β-lap induces a non-caspase-mediated pathway or that zVAD-fmk cannot inhibit this particular caspase-mediated pathway (Fig. 4B).

Cleavage of lamin B (60-kDa full-length protein) to a characteristic 46-kDa polypeptide, typically by caspase 6, is believed to aid in the breakdown of the architecture necessary for apoptosis-related nuclear condensation and membrane blebbing [48–50]. Cleavage of lamin B in β-lap-treated MCF-7:WS8 cells was noted [3]. In DU-145 cells, β-lap but not CPT treatment resulted in lamin B cleavage, possibly due to the relatively poor apoptotic responses induced by CPT compared to those induced by β-lap. Interestingly, 100 μM zVAD-fmk, the pan-caspase inhibitor, did not inhibit β-lap-mediated cleavage of lamin B (Fig. 4B). These data are consistent with prior data from our laboratory that β-lap can stimulate a non-caspase-mediated, cysteine protease-directed apoptotic pathway in certain human cancer cells [35].

We previously showed that p53 was not necessary for β-lap-induced apoptosis [1]. In fact, we reported that the level of p53 *decreased* following treatment of wild-type p53-expressing MCF-7 breast cancer cells following 4–10 μM β-lap [3]. In mutant p53-expressing DU-145 cells, β-lap treatment resulted in the formation of two cleavage fragments (40 kDa and ~20 kDa) that were not inhibited by 100 μM zVAD-fmk coadministration (Fig. 4B). A similar cleavage of p53 was described during calpain-mediated apoptosis, and this protease may be involved in β-lap-mediated cell death responses [35]. Treatment of DU-145 cells with CPT did not result in any changes in the level or cleavage of p53, even though 20% of the cells were apoptotic; DU-145 cells express stable, high levels of mutant p53 protein that are not stabilized by CPT-mediated damage.

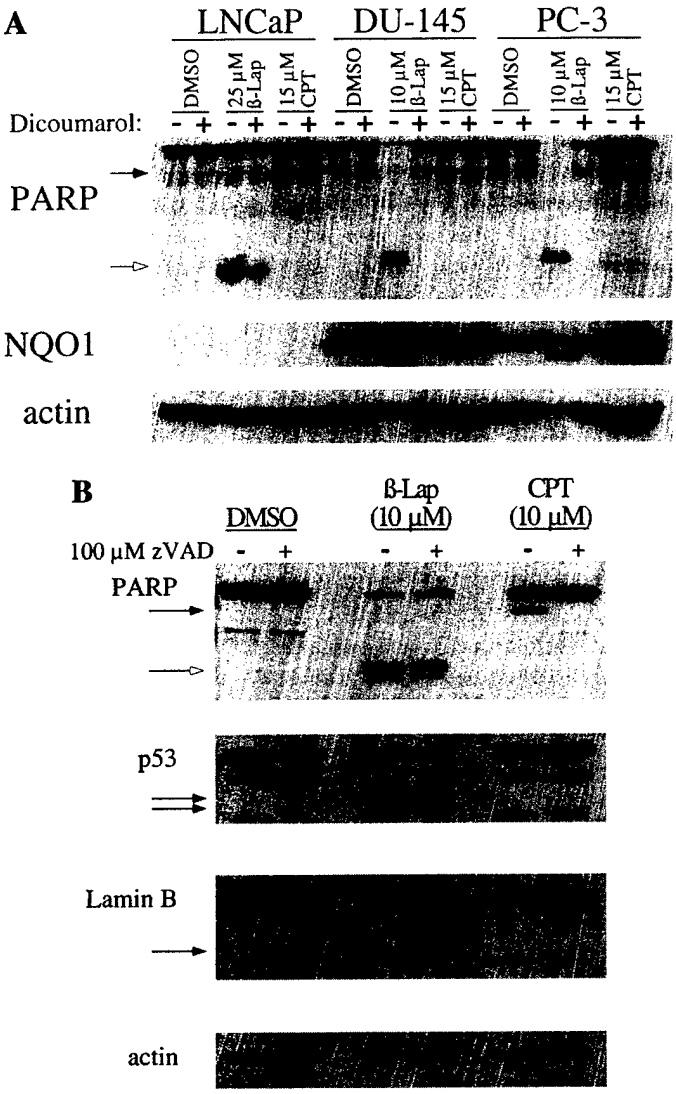

FIG. 4. (A) PARP cleavage in human prostate cancer cells following β-lap or CPT exposure. Human prostate cancer cell lines were treated for 4 h with β-lap (10 or 25 μM) or CPT (15 μM), with or without 50 μM dicoumarol. Cells were harvested for analyses 24 h posttreatment and analyzed for specific changes in protein cleavage events by Western blot analyses. Closed arrow; typical 89-kDa PARP cleavage fragment. Open arrow, atypical 60-kDa PARP cleavage fragment. (B) zVAD-fmk blocks CPT-, but not β-lap-, induced apoptotic proteolytic substrate cleavage in DU-145 cells. DU-145 human prostate cancer cells were treated with either 10 μM β-lap or 10 μM CPT, with or without 100 μM zVAD-fmk, for 4 h and specific protein cleavage events were monitored by Western immunoblot analyses. zVAD-fmk treatment began 1 h prior to β-lap addition; treatment was continued throughout the β-lap treatment, and cells were harvest as described under Materials and Methods. PARP: full-length polypeptide, 113 kDa; typical PARP cleavage fragment (closed arrow), 89 kDa; atypical PARP cleavage fragment (open arrow), ~60 kDa. p53: full-length polypeptide, 53 kDa; p53 cleavage fragment, ~40 kDa. Lamin B: full-length polypeptide, 68 kDa; lamin B cleavage fragment, 45 kDa.

Stable transfection of LNCaP cells with NQO1. In order to further characterize the role of NQO1 in β-lap-mediated apoptosis, LNCaP cells were transfected with either pcDNA3 empty vector or pcDNA3 containing full-length NQO1 cDNA, in which expression of this two-electron reductase was controlled by the CMV promoter. Five clonal cell lines containing NQO1 (LN-NQ Clones 1–4, 10) and one vector-alone control (LN-pcDNA3) were isolated. All five NQO1-containing cell lines demonstrated both enzyme activity (15- to 30-fold above nontransfected levels, Table 1) and protein expression (Fig. 7). LNCaP transfectants containing pcDNA3 vector alone exhibited neither NQO1 enzyme activity nor protein expression, similar to nontransfected LNCaP parental cells (Table 1, Fig. 7).

Transfection of NQO1 sensitized human LNCaP prostate cancer cells to β-lap. In clonogenic assays, NQO1-deficient parental LNCaP cells showed moderate resistance to β-lap, relative to DU-145 and PC-3 cells, which express high levels of the enzyme (Fig. 2). Similarly, NQO1-containing LNCaP clones demonstrated significantly increased sensitivity to β-lap relative to NQO1-deficient LNCaP cells containing pcDNA3 vector alone (Fig. 5A). As previously observed with NQO1-expressing DU-145 or PC-3 cells, coadministration of dicoumarol blocked β-lap-mediated cytotoxicity. This resulted in a relatively resistant phenotype, similar to that of NQO1-deficient, pcDNA3 vector-alone, control LNCaP cells. Dicoumarol coadministration had no effect on the sensitivity of NQO1-deficient, LNCaP cells (containing pcDNA3 vector alone) to β-lap treatment (Fig. 5A).

Menadione is detoxified by NQO1 and is thus toxic to cells in the absence of NQO1 activity. In contrast to β-lap-mediated toxicity, NQO1-deficient LNCaP parental or vector-alone transfectants were more sensitive to menadione on an equimolar basis. NQO1-containing LN-NQ clone 10 cells were more resistant to menadione toxicity than NQO1-deficient LN-pcDNA3 cells (Fig. 5B). Thus, the toxicities of menadione and β-lap were reversed. Similar results were found with human NQO1-transfected (or vector-alone-transfected) MDA-MB-468 breast cancer cells treated with β-lap or menadione [8].

To determine whether LNCaP cells compensated for their NQO1 deficiency by increasing the activities of one-electron enzymes, levels of P450 reductase and cytochrome b5R were determined in the three parental cell lines, as well as in the six NQO1-expressing LNCaP clones. No significant differences in P450 reductase or b5R activities were noted (Table 1).

Transfection of LNCaP cells with NQO1 enhanced β-lap-induced apoptosis. Exposure of each NQO1-expressing LNCaP transfectant (LN-NQ Clones 1–4, 10) to 10 μM β-lap resulted in significantly increased apoptosis (i.e., 80–90%) compared to that of control LNCaP transfectants containing pcDNA3 vector alone (5%) (Fig. 6). As expected, NQO1-mediated, β-lap-stimulated apoptosis in LN-NQ Clone 1–4 and 10 cell lines were prevented by 50 μM dicoumarol.

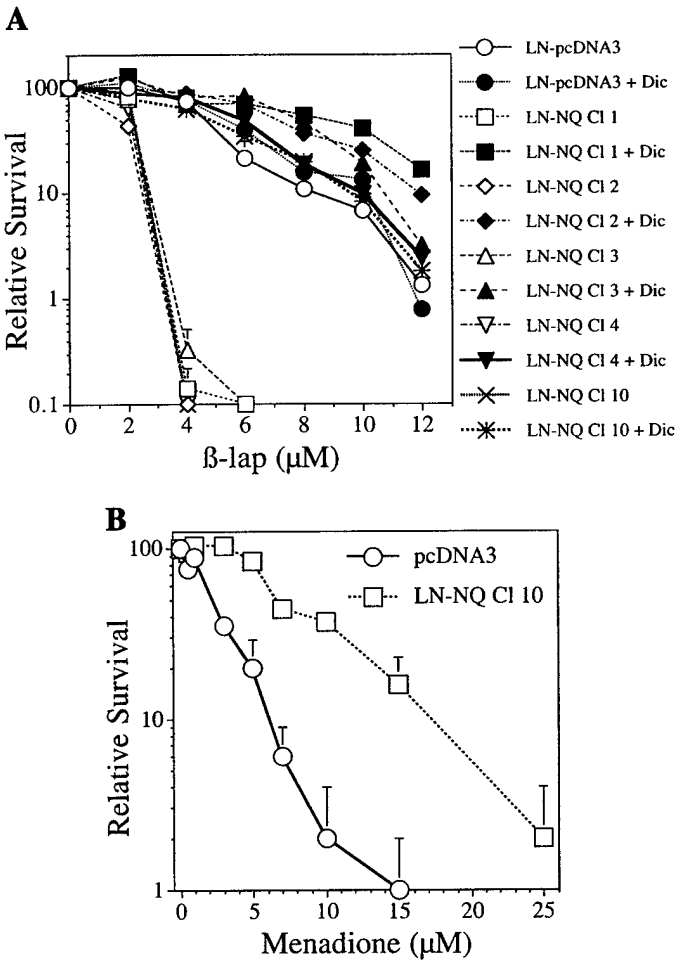

FIG. 5. (A) Transfection of LNCaP cells with NQO1 enhances β-lap-induced lethality. NQO1-containing (LN-NQ Clones 1–4, 10) and -deficient (LN-pcDNA3) LNCaP clonal cell lines were treated with 4-h pulses of various doses of β-lap, with or without concomitant 50 μM dicoumarol coadministration. Survival was then determined by colony-forming-ability assays as described under Materials and Methods. Experiments were performed three times, each in triplicate. Symbols represent means ± SD. Open symbols, β-lap alone. Closed symbols, β-lap with 50 μM dicoumarol coadministration. (B) Transfection of LNCaP cells with NQO1 decreases menadione-induced lethality. One NQO1-transfected LNCaP clonal cell line (LN-NQ Cl 10) and the LNCaP vector-alone clonal isolate (LN-pcDNA3) were treated with 4-h pulses of various doses of menadione, and survival was determined by CFA assays as described under Materials and Methods. Experiments were performed three times, each in triplicate. Symbols represent means ± SD.

Expression of NQO1 in LNCaP cells enhanced atypical PARP cleavage in response to β-lap exposure. Parental LNCaP cells produced an apoptosis-related, atypical cleavage of PARP (formation of a 60-kDa

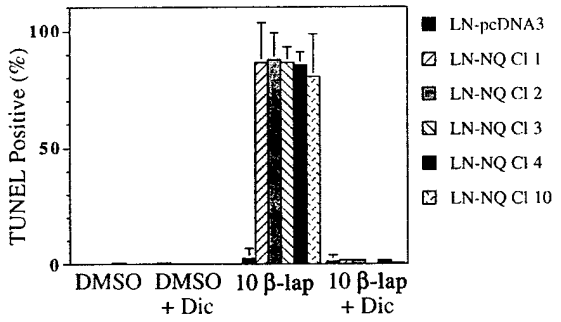

FIG. 6. Stable transfection of LNCaP cells with NQO1 enhances β-lap-induced apoptosis. Stably transfected LNCaP clonal cell lines containing NQO1 or vector alone (from Fig. 5A) were treated for 4 h with various concentrations of β-lap, with or without 50 μM dicoumarol, as described under Materials and Methods. Forty-eight hours posttreatment, cells were monitored for apoptosis-related DNA fragmentation using TUNEL assays. Symbols represent means ± SD of experiments performed three or more times, each in triplicate. LNCaP isolated clonal cell lines examined were pcDNA3, LNCaP stably transfected with vector alone; LN-NQ Cl 1–4 and 10, five separate LNCaP cell lines stably transfected with CMV-controlled NQO1 cDNA, Clones 1–4 and 10.

PARP polypeptide) following 25 μM β-lap, a concentration nearly five times higher than the LD_{50} for the drug (see Figs. 2 and 4A). In contrast, atypical PARP cleavage was apparent in PC-3 or DU-145 cells after 10 μM β-lap, at or near the drug's LD_{90} for these cells. In general, atypical PARP cleavage correlated well with the sensitivities (apoptosis) of each prostate cancer cell line to β-lap exposure (Figs. 2 and 4A), similar to that observed in human breast carcinoma epithelial cells [35]. Empty vector (LN-pcDNA3)- or NQO1-transfected LNCaP (LN-NQ Clones 1–4 and 10) cells were also examined for PARP cleavage following β-lap treatments. Atypical 60-kDa PARP fragmentation was observed in each NQO1-expressing clone following 10 μM β-lap, whereas the parental and vector-alone clones needed significantly greater doses of the compound (≥25 μM) to initiate detectable levels of PARP cleavage (Fig. 7). Thus, PARP fragmentation in NQO1-containing LNCaP cells, but not in LNCaP parental or empty vector transfectants, following β-lap treatment strongly correlated with overall apoptosis (Fig. 6) and lethality (Fig. 5A). In contrast, altered expression of NQO1 did not influence apoptotic reactions induced by CPT in any of the LNCaP cell lines examined above.

DISCUSSION

NQO1 may be a clinically exploitable target for therapy against certain tumors using β-lap or its derivatives. Our results demonstrate that NQO1 is a key intracellular determinant for β-lap toxicity in human prostate epithelial cancer cells, since dicoumarol prevented β-lap-mediated apoptosis and lethality in DU-145 and PC-3. In contrast, dicoumarol did not affect β-lap-induced apoptosis in NQO1-deficient LNCaP cells. Furthermore, reexpression of NQO1 in deficient LNCaP cells increased their sensitivity to β-lap-mediated apoptosis and lethality. These data suggest that NQO1 activity is a key determinant in β-lap-mediated cytotoxicity, a conclusion also made using human breast cancer cells [8]. Although many laboratories (including our own) have published data supporting other potential targets *in vitro,* including Topo I and Topo II-α, none of these previous studies demonstrated convincing data for an intracellular target for this drug.

We previously showed that β-lap induced a p53-independent apoptotic response in human prostate cancer cells [37]. We now demonstrate that these p53-independent apoptotic responses initiated by this drug are greatly enhanced by NQO1 expression (Fig. 7). Furthermore, we demonstrate that lethality caused by β-lap is opposite to that induced by menadione, wherein NQO1 overexpression increases β-lap lethality but decreases the cytotoxicity of menadione. Similar results were found in human breast cancer cells [8]. Collectively, our data strongly suggest that β-lap is bioactivated in cells expressing NQO1. The possibility of a bioactivated form of β-lap interacting with previ-

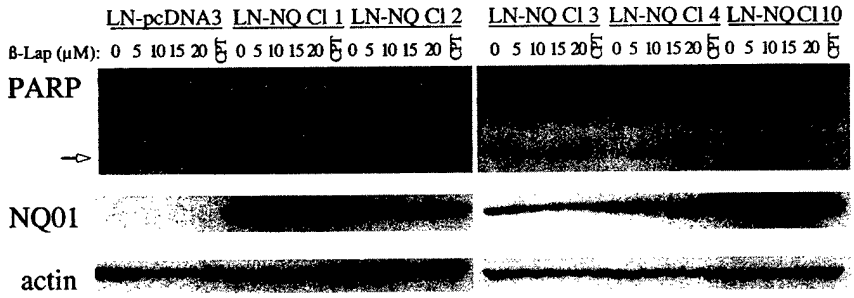

FIG. 7. β-Lap-induced atypical PARP cleavage is enhanced by NQO1 overexpression. NQO1-containing and -deficient LNCaP clonal cell lines (described in the legends to Figs. 5A and 6), were exposed to 4-h treatments with various doses of β-lap or 10 μM CPT. Cells were harvested for Western immunoblot analyses 24 h following drug removal, as previously described. Open arrow, atypical PARP cleavage fragment of ~60-kDa molecular weight by SDS–PAGE.

ously suggested *in vitro* targets, such as Topo I [2], is being explored.

β-Lap induces a unique apoptotic response in epithelial cancer cell lines, such as those of breast or prostate origin. β-Lap stimulates a novel cell death pathway that appears to be caspase-independent (Fig. 4B), calcium-dependent, and NQO1-mediated (Fig. 7) [8, 35]; dicoumarol prevents its activation and cells lacking NQO1 do not demonstrate p53 or PARP proteolytic cleavage events after physiological β-lap exposures (non-supra-lethal doses) (Fig. 4A) [8]. Treatment of human prostate cancer cells with β-lap induced the formation of an atypical PARP cleavage fragment, different from the classical 89-kDa fragment formed during caspase-mediated (via caspases 3, 6, and 7) apoptosis [34]. Production of this atypical 60-kDa PARP fragment correlated well with apoptosis and overall sensitivity of human prostate or breast epithelial cancer cells to β-lap (compare Figs. 5A and 7) [8]. As with β-lap-treated human breast cancer cells, although the overall number of adherent cells was markedly reduced, no evidence of cell *lysis* during β-lap-mediated apoptosis in DU-145 or PC-3 cells was noted, suggesting that cell death was not necrotic in nature. Addition of 100 μM zVAD-fmk, a widely used pan-caspase inhibitor, blocked caspase-induced typical PARP cleavage initiated in DU-145 cells by treatment with 10 μM CPT (Fig. 3B). However, the same concentration of zVAD-fmk had no effect on atypical PARP cleavage or cleavage of other β-lap-induced apoptotic substrates, such as lamin B or p53, in NQO1-expressing human prostate cancer cell lines. Thus, β-lap predominantly stimulates a non-caspase-mediated apoptotic response, which we theorize is directed by the activation of a calcium-dependent cysteine protease with properties similar to calpain [35].

LNCaP cells did exhibit toxicity following nonphysiologically high doses of β-lap, despite their deficiency in NQO1 expression. The observed apoptotic responses in LNCaP parental cells following supralethal doses of β-lap are probably attributed to the lower affinity reduction of this compound by one-electron reducing enzymes, such as p450 reductase, as well as other non-related enzymes (e.g., cytochrome b5 reductase). These enzymes may catalyze two-step, one-electron reductions of quinones (i.e., β-lap) in order to form the hydroquinone, whereas NQO1 mediates one higher affinity, two-electron reduction forming the same byproduct. As a result, a higher dose of β-lap was required (compared to NQO1-containing PC-3, DU-145, or LNCaP transfectants) for a similar apoptotic reaction. Expression of NQO1 in LNCaP cells, via stable transfection with CMV-controlled mammalian NQO1 expression vectors, *significantly* increased their sensitivity to β-lap, a sensitivity ablated by dicoumarol coadministration. These data indicate that while NQO1 is not the only enzyme capable of activating or metabolizing β-lap, its ability far surpasses the efficiency of other reductases (or other as yet unidentified enzymes) in the cell to bioactivate the drug.

Current dogma states that all apoptotic pathways include caspase activation and that all caspase-independent mechanisms lead exclusively to necrosis. Our data strongly suggest that other non-caspase-mediated apoptotic pathways (e.g., mediated by calpain) are activated after certain drug treatments. Non-caspase-mediated apoptotic pathways have been described in several other cell systems [51–56]. Furthermore, we suggest that there is a spectrum of cell death responses, ranging from caspase-mediated apoptosis to cell lysis during necrosis (i.e., cell plasma membrane rupture and lysis, as observed after sodium azide exposure). Cells treated with β-lap exhibit many characteristics of cells undergoing apoptosis, including morphologic changes (Fig. 3A); chromatin condensation [63]; DNA ladder formation [14, 20]; generation of sub-G_0/G_1 apoptotic cells [1]; cells staining positive with the TUNEL assay, which monitors for endonuclease-specific DNA double-strand breaks (Fig. 3B, 6); specific dephosphorylation of pRb [3]; and specific intracellular cleavage of unique substrates (e.g., Topo I, Topo II, lamin B, and p53), while most other proteins (e.g., cyclins A, B, E and bcl-2) remained intact (Figs. 4A, 4B, and 7) [3, 35]. Yet, concrete evidence of caspase activation is lacking. It was previously reported that β-lap induced apoptosis in some cell systems and necrosis in others, although specific end points for necrosis were not examined [57]. β-Lap-treated breast or prostate cancer cells demonstrated extensive formation of apoptotic cells, as monitored by TUNEL assays, formation of sub-G_0/G_1 cells, morphology changes (i.e., condensed nuclei and rounded cells), and lamin B cleavage (Fig. 4B), as early as 4–8 h following β-lap treatment [8, 35]. Since DNA fragmentation may occur during late-stage necrosis [58], the early (4–8 h) appearance of cells staining positive using a TUNEL assay, concomitant with specific protein cleavage events (e.g., PARP and p53) following β-lap treatment, strongly suggests that an apoptotic, rather than necrotic, cell death mechanism was triggered by β-lap. β-Lap-treated NQO1-expressing cells demonstrate extensive nuclear condensation and unique intracellular substrate cleavages, and the cells detached in a rounded form (Fig. 3). Most importantly, β-lap-treated cells show no visible morphologic hallmarks of necrosis, such as extensive cell debris (Fig. 3). Few cells survive the treatment and the cytotoxic responses have a sharp dose-response curve in which apoptosis and loss of survival are directly correlated in NQO1-expressing cells. All NQO1-containing breast and prostate cancer cells examined thus far respond with identical apoptotic mechanisms to the drug. In contrast, all NQO1-deficient breast or prostate

cancer cells appear to be more resistant to β-lap, showing significantly less apoptosis [8].

We have previously shown that β-lap was a radiosensitizer (after IR exposure) of human cancer cells compared to normal cells [2]. Furthermore, those normal cells that did survive the IR exposures plus β-lap posttreatments demonstrated lower than basal levels of neoplastic transformants [59]. Our laboratory also demonstrated that NQO1 was an X-ray-inducible transcript (i.e., xip3) [10]. The discovery that NQO1 is a major determinant in the sensitivity of human prostate and breast epithelial cancer cells to β-lap [8] may explain the compound's ability to radiosensitize certain cancer cells that express low basal levels of NQO1, but in which the cell's enzyme levels can be dramatically induced by IR pretreatment. We previously found that posttreatments, and not pretreatments, of β-lap sensitized cells to IR [2, 59–61]. A 5-h posttreatment of 4–5 μM β-lap was required, in which IR-treated cells were killed and non-IR-treated cells were spared (<20% lethality). Since NQO1 levels were induced 5- to 20-fold in 3 to 4 h in the same cell line [59], we speculate that the compound's radiosensitizing capacity was due to the exploitation of this damage-inducible, bioactivating (for β-lap) enzyme. Since NQO1 is commonly elevated during early stages of carcinogenesis [62, 63], normal cells that become genetically unstable following IR exposure and later induce stable expression of NQO1 would be rather sensitive to cell death by β-lap posttreatments. We previously demonstrated that post-IR-exposure to 4–5 μM β-lap could dramatically reduce IR-mediated neoplastic transformants [59]. We speculate, therefore, that this compound not only may be useful against NQO1-overexpressing cancer cells (e.g., breast, lung, and possibly prostate cancers), but also could possess great potential as an anti-carcinogenic agent by eliminating genetically unstable, NQO1-overexpressing transformed cells within a normal cell population.

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Calcium Is a Key Signaling Molecule in β-Lapachone-mediated Cell Death*

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β-Lapachone (β-Lap) triggers apoptosis in a number of human breast and prostate cancer cell lines through a unique apoptotic pathway that is dependent upon NQO1, a two-electron reductase. Downstream signaling pathway(s) that initiate apoptosis following treatment with β-Lap have not been elucidated. Since calpain activation was suspected in β-Lap-mediated apoptosis, we examined alterations in Ca^{2+} homeostasis using NQO1-expressing MCF-7 cells. β-Lap-exposed MCF-7 cells exhibited an early increase in intracellular cytosolic Ca^{2+}, from endoplasmic reticulum Ca^{2+} stores, comparable to thapsigargin exposures. 1,2-Bis-(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester, an intracellular Ca^{2+} chelator, blocked early increases in Ca^{2+} levels and inhibited β-Lap-mediated mitochondrial membrane depolarization, intracellular ATP depletion, specific and unique substrate proteolysis, and apoptosis. The extracellular Ca^{2+} chelator, EGTA, inhibited later apoptotic end points (observed >8 h, *e.g.* substrate proteolysis and DNA fragmentation), suggesting that later execution events were triggered by Ca^{2+} influxes from the extracellular milieu. Collectively, these data suggest a critical, but not sole, role for Ca^{2+} in the NQO1-dependent cell death pathway initiated by β-Lap. Use of β-Lap to trigger an apparently novel, calpain-like-mediated apoptotic cell death could be useful for breast and prostate cancer therapy.

β-Lap[1] is a naturally occurring compound present in the bark of the South American Lapacho tree. It has antitumor activity against a variety of human cancers, including colon, prostrate, promyelocytic leukemia, and breast (1–3). β-Lap was an effective agent (alone and in combination with taxol) against human ovarian and prostate xenografts in mice, with little host toxicity (4). We recently demonstrated that β-Lap kills human breast and prostate cancer cells by apoptosis, a cytotoxic response significantly enhanced by NAD(P)H:quinone oxidoreductase (NQO1, E.C. 1.6.99.2) enzymatic activity (5).[2] β-Lap cytotoxicity was prevented by co-treatment with dicumarol (an NQO1 inhibitor) in NQO1-expressing breast and prostate cancer cells (5).[2] NQO1 is a cytosolic enzyme elevated in breast cancers (6) that catalyzes a two-electron reduction of quinones (*e.g.* β-Lap, menadione), utilizing either NADH or NADPH as electron donors. Reduction of β-Lap by NQO1 presumably leads to a futile cycling of the compound, wherein the quinone and hydroquinone form a redox cycle with a net concomitant loss of reduced NAD(P)H (5).

Apoptosis is an evolutionarily conserved pathway of biochemical and molecular events that underlie cell death processes involving the stimulation of intracellular zymogens. The process is a genetically programmed form of cell death involved in development, normal turnover of cells, and in cytotoxic responses to cellular insults. Once apoptosis is initiated, biochemical and morphological changes occur in the cell. These changes include: DNA fragmentation, chromatin condensation, cytoplasmic membrane blebbing, cleavage of apoptotic substrates (*e.g.* PARP, lamin B), and loss of mitochondrial membrane potential with concomitant release of cytochrome c into the cytoplasm (7–9). Apoptosis is a highly regulated, active process that requires the participation of endogenous cellular enzymes that systematically dismantle the cell. The most well characterized proteases in apoptosis are caspases, aspartate-specific cysteine proteases, that work through a cascade that can be initiated by mitochondrial membrane depolarization leading to the release of cytochrome *c* and Apaf-1 into the cytoplasm (10), that then activates caspase 9 (11). Non-caspase-mediated pathways are less understood.

We previously showed that apoptosis following β-Lap administration was unique, in that an ~60-kDa PARP cleavage fragment, as well as distinct intracellular proteolytic cleavage of p53, were observed in NQO1-expressing breast or prostate cancer cells (5).[2] These cleavage events were distinct from those observed when caspases were activated by topoisomerase I poisons, staurosporine, or administration of granzyme B (5, 12, 13). Furthermore, β-Lap-mediated cleavage events were blocked by administration of global cysteine protease inhibitors, as well as extracellular Ca^{2+} chelators (12). Based on these data, we concluded that β-Lap exposure of NQO1-expressing breast and prostate cancer cells caused the activation of a Ca^{2+}-dependent protease with properties similar to calpain; in particular, the p53 cleavage pattern of β-Lap-exposed

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[1] The abbreviations used are: β-Lap, β-lapachone; MCP, MCF-7:WS8; NQO1, NAD(P)H:quinone oxidoreductase, DT-diaphorase (E.C. 1.6.99.2); PARP, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; ER, endoplasmic reticulum; TG, thapsigargin; STS, staurosporine; BAPTA-AM, 1,2-bis-(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester.

[2] S. M. Planchon, C. Tagliarino, J. J. Pink, W. G. Bornmann, M. E. Varnes, and D. A. Boothman. *Exp. Cell Res.*, in press.

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cells was remarkably similar to the pattern observed after calpain activation (14, 15).

Ca^{2+} is recognized as an important regulator of apoptosis (16–21). The cytoplasmic Ca^{2+} concentration is maintained at ~100 nM in resting cells by relatively impermeable cell membranes, active extrusion of Ca^{2+} from the cell by plasma membrane Ca^{2+}-ATPases, plasma membrane Na^+/Ca^{2+} exchangers, and active uptake of cytosolic Ca^{2+} into the endoplasmic reticulum (ER) by distinct Ca^{2+}-ATPases. In contrast, the concentration of Ca^{2+} in the extracellular milieu and in the ER is much higher (in the millimolar range). Evidence for involvement of Ca^{2+} influx into the cytosol as a triggering event for apoptosis has come from studies with specific Ca^{2+} channel blockers that abrogate apoptosis in regressing prostate following testosterone withdrawal (22). Other support for the involvement of Ca^{2+} in apoptosis comes from the observation that agents that directly mobilize Ca^{2+} (*e.g.* Ca^{2+} ionophores or the sarcoplasmic reticulum Ca^{2+}-ATPase pump inhibitor, thapsigargin, TG) can trigger apoptosis in diverse cell types (23–27). Inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase pump by TG causes a transient increase in cytoplasmic Ca^{2+} from ER Ca^{2+} stores, and a later influx of Ca^{2+} from the extracellular milieu, leading to the induction of apoptotic cell death (24, 27, 28). Consequently, emptying of intracellular Ca^{2+} stores may trigger apoptosis by disrupting the intracellular architecture and allowing key elements of the effector machinery (*e.g.* Apaf-1) to gain access to their substrates (*e.g.* caspase 9). Ca^{2+} has also been shown to be necessary for apoptotic endonuclease activation, eliciting DNA cleavage after many cellular insults (29–31). Buffering intracellular Ca^{2+} released from stored Ca^{2+} pools (*e.g.* ER) with BAPTA-AM, or removal of extracellular Ca^{2+} with EGTA, can protect cells against apoptosis (32, 33). Therefore, increases in intracellular Ca^{2+} levels appear to be important cell death signals in human cancer cells that might be exploited for anti-tumor therapy. Finally, Ca^{2+} may act as a signal for apoptosis by directly activating key proapoptotic enzymes (*e.g.* calpain); however, these proteolytic responses are poorly understood. The role of Ca^{2+} in cell death processes involving caspase activation has been examined in detail (28, 34–36). However, the role of Ca^{2+} in non-caspase-dependent cell death responses is relatively unexplored.

Recent studies have suggested that alterations in mitochondrial homeostasis play an essential role in apoptotic signal transduction induced by cytotoxic agents (37, 38). Various apoptotic stimuli have been shown to induce mitochondrial changes, resulting in release of apoptogenic factors, apoptosis-inducing factor (39), and mitochondrial cytochrome *c* (9) into the cytoplasm. These changes are observed during the early phases of apoptosis in human epithelial cells, and were linked to the initial cascade of events, sending the cell to an irreversible suicide pathway. During high, sustained levels of cytosolic Ca^{2+}, mitochondrial Ca^{2+} uptake is driven by mitochondrial membrane potential to maintain Ca^{2+} homeostasis in the cytosol. In de-energized mitochondria, Ca^{2+} can be released by a reversal of this uptake pathway (40). These data, therefore, linked changes in Ca^{2+} homeostasis and mitochondrial membrane potential to the initiation of apoptosis. Li *et al.* (41) reported that β-Lap caused a decrease in mitochondrial membrane potential with release of cytochrome *c* into the cytoplasm in a number of human carcinoma cell lines, shortly after drug addition. Other alterations in metabolism (*e.g.* ATP depletion) have not been examined in β-Lap-treated cells.

We previously characterized the activation of a novel cysteine protease in various breast cancer cell lines with properties similar to the Ca^{2+}-dependent cysteine protease, calpain, after exposure to β-Lap (12). Using NQO1-expressing breast cancer cells, we show that β-Lap elicits a rise in intracellular Ca^{2+} levels shortly after drug administration that eventually leads to apoptosis. This paper suggests a critical, but not sufficient, role for Ca^{2+} in the cell death pathway initiated by NQO1-dependent bioactivation of β-Lap. Possible combinatorial effects (*e.g.* NAD(P)H depletion as well as intracellular calcium alterations) that initiate β-Lap-mediated apoptosis in NQO1-expressing breast cancer cells will be discussed.

EXPERIMENTAL PROCEDURES

Reagents—β-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2b]pyran-5,6-dione) was synthesized by Dr. William G. Bornmann (Memorial Sloan Kettering, New York), dissolved in dimethyl sulfoxide at 10 mM, and the concentration verified by spectrophotometric analysis (2, 5). EGTA, Hoescht 33258, and thapsigargin were obtained from Sigma. BAPTA-AM (1,2-bis-(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetra-(acetoxymethyl ester)) was obtained from Calbiochem (La Jolla, CA). JC-1 (5,5'6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) and Fluo-4-AM were obtained from Molecular Probes, Inc. (Eugene, OR).

Cell Culture—MCF-7:WS8 (MCF-7) human breast cancer cells were obtained from Dr. V. Craig Jordan, (Northwestern University, Chicago, IL). MDA-MB-468 cells were obtained from the American Type Culture Collection and transfected with NQO1 cDNA in the pcDNA3 constitutive expression vector as described previously (5). Tissue culture components were purchased from Life Technologies, Inc., unless otherwise stated. MCF-7 cells were grown in RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum, in a 37 °C humidified incubator with 5% CO_2, 95% air atmosphere as previously described (2, 5). For all experiments, log-phase breast cancer cells were exposed to 5 μM β-Lap for 4 h (unless otherwise indicated), after which fresh medium was added and cells were harvested at various times post-treatment.

TUNEL Assay—Cells were seeded at 1×10^6 cells/10-cm Petri dish and allowed to grow for 24 h. Log-phase cells were then pretreated for 30 min with 10 μM BAPTA-AM, 3 mM EGTA, or 50 μM dicumarol followed by a 4-h pulse of 5 μM β-Lap, as described above, or 24 h treatment of 10 μM ionomycin or 1 μM staurosporine. Medium was collected from experimental as well as control conditions 24 h later, and attached along with floating cells were monitored for apoptosis using TUNEL 3'-biotinylated DNA end labeling via the APO-DIRECT kit (Pharmingen, San Diego, CA) as described (5). Apoptotic cells were analyzed and quantified using an EPICS XL-MCL flow cytometer that contained an air-cooled argon laser at 488 nm, 15 mW (Beckman Coulter Electronics; Miami, Fl), and XL-MCL acquisition software provided with the instrument.

Cell Growth Assays—MCF-7 cells were seeded at 5×10^4 cells per well in a 12-well plate and allowed to attach overnight. The following day, log-phase cells were pretreated for 30 min with 5 μM BAPTA-AM, followed by a 4-h pulse of β-Lap (0–5 μM). Drugs were removed and fresh medium added. Cells were allowed to grow for an additional 6 days. DNA content (a measure of cell growth) was determined by fluorescence using Hoechst dye 33258 as described (5) and changes in growth were monitored using a PerkinElmer HTS 7000 Plus Bio Assay Plate Reader (Norwalk, CT) with 360 and 465 nm excitation and emission filters, respectively. Data were expressed as relative growth, T/C (treated/control), using experiments performed at least twice.

Confocal Microscopy—MCF-7 cells were seeded at $2–3 \times 10^5$ cells per 35-mm glass bottom Petri dishes (MatTek Corp., Ashland, MA) and allowed to attach overnight. Cells were rinsed twice in a Ca^{2+}/Mg^{2+} balanced salt solution (BSS, 130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM HEPES, pH 7.5, 5 mM glucose, 1 mg/ml bovine serum albumin) and loaded with the Ca^{2+}-sensitive fluorescent indicator, fluo-4-AM (5 μM), in BSS for ~20–30 min at 37 °C. Cells were rinsed twice in BSS and incubated for an additional 20 min at 37 °C to allow for hydrolysis of the AM-ester. Cells were imaged with a Zeiss 410 confocal microscope (Thornwood, NY) equipped with a ×63 N.A. 1.4 oil immersion planapochromat objective at room temperature (the same results were observed at room temperature and 37 °C). Confocal images of fluo-4 fluorescence were collected using a 488-nm excitation light from an argon/krypton laser, a 560-nm dichroic mirror, and a 500–550 nm band-pass barrier filter. Three basal images were collected before drug addition (8 μM β-Lap, ± 50 μM dicumarol or 200 nM TG). The mean pixel intensity was set to equal one for analyses of fold-increase in fluo-4 fluorescence intensity. Subsequently, images were collected after the indicated treatments at 90-s intervals. BAPTA-AM (20 μM) was co-

loaded with fluo-4-AM where indicated. Mean pixels were determined in regions of interest for individual cells at each time point.

Mitochondrial Membrane Potential Determinations—MCF-7 cells were seeded at 2.5–3 × 10^5 cells per 6-well plate, and allowed to grow for 24 h. Log-phase cells were pretreated for 30 min with 10 μM BAPTA-AM, 3 mM EGTA, or 50 μM dicumarol followed by a 4-h pulse of 5 μM β-Lap, unless otherwise indicated. Cells were trypsinized and resuspended in phenol red-minus RPMI medium for analyses. Cells were maintained at 37 °C for the duration of the experiment, including during analyses. Prior to analyses, cells were loaded with 10 μg/ml JC-1 for 9–14 min and samples were analyzed using a Beckman Coulter EPICS Elite ESP (Miami, FL) flow cytometer. JC-1 monomer and aggregate emissions were excited at 488 nM and quantified using Elite acquisition software after signal collection through 525- and 590-nm band pass filters, respectfully. Shifts in emission spectra were plotted on bivariant dot plots, on a cell-by-cell basis, to determine relative mitochondrial membrane potential of treated and control cells.

ATP Measurements—Cells were seeded at 2.5 × 10^5 cells per well in 6-well dishes and allowed to attach for 24 h. Fresh medium was added to the cells along with Ca^{2+} chelators or dicumarol 30 min prior to β-Lap exposure (4 h unless otherwise indicated). Floating cells were collected, pelleted, and lysed in 1.67 M perchloric acid. Attached cells were lysed directly in 1.67 M perchloric acid. Following a 20-min incubation at room temperature, attached cells were scraped and transferred to corresponding microcentrifuge tube, cooled on ice for several minutes, and spun to pellet protein precipitates. Deproteinized samples were neutralized with 3.5 M KOH and HEPES/KOH (25 mM HEPES, 15 mM KOH, pH 8), and incubated on ice for 15 min. Precipitates were removed by centrifugation and samples stored at −20 °C. Cell extracts were analyzed for ATP and ADP levels using a luciferase-based bioluminescent assay and rephosphorylation protocols, as described (42).

Western Blot Analyses—Whole cell extracts from control or β-Lap-exposed MCF-7 cells were prepared and analyzed by SDS-polyacrylamide gel electrophoresis/Western blot analyses as previously described (2, 5, 12). Loading equivalence and transfer efficiency were monitored by Western blot analyses of proteins that are known to be unaltered by experimental treatments (2), and using Ponceau S staining of the membrane, respectively. Probed membranes were then exposed to x-ray film for an appropriate time and developed. Dilutions of 1:10,000 for the C-2–10 anti-PARP antibody (Enzyme Systems Products, Livermore, CA), and 1:2000 for anti-p53 DO-1 and anti-lamin B (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used as described (2, 12).

RESULTS

Ca^{2+} Chelators Prevent β-Lap-induced Apoptotic DNA Fragmentation and Protect against Cell Death—Log-phase MCF-7 cells were treated for 4 h with 5 μM β-Lap, fresh medium was then applied, and cells were harvested 24 h later and analyzed for DNA fragmentation (*i.e.* apoptotic cells staining positive in a TUNEL assay). Treatment of MCF-7 cells with β-Lap resulted in >90% apoptotic cells (Fig. 1, *A* and *B*). However, MCF-7 cells exposed to a 30-min pretreatment with 10 μM BAPTA-AM or 3 mM EGTA, followed by a 4-h pulse of 5 μM β-Lap, exhibited only 20 or 39% apoptotic cells, respectively, in 24 h.

To examine whether BAPTA-AM could affect β-Lap lethality, we measured relative growth of MCF-7 cells with or without exposure to β-Lap, and in the presence or absence of BAPTA-AM. MCF-7 cells were treated for 30 min with 5 μM BAPTA-AM, subsequently exposed to a 4-h pulse of β-Lap (1.5−5 μM), and relative cell growth was measured 6 days later (Fig. 1*C*). The LD_{50} dose of β-Lap in MCF-7 cells was ~2.5 μM in colony forming assays, which correlated well with IC_{50} relative growth inhibition, as measured by DNA content (2, 5). At 1.5 μM β-Lap, cells exhibited little or no toxicity. At β-Lap doses of 3 or 5 μM, cells exhibited considerable toxicity, >90% growth inhibition, as previously reported (2, 5). Toxicity was significantly prevented by 5 μM BAPTA-AM pretreatment. BAPTA-AM pretreated cells exhibit only 44 and 73% growth inhibition after 3 or 5 μM β-Lap treatments, respectively (Fig. 1*C*). BAPTA alone did not affect MCF-7 cell growth compared with untreated controls.

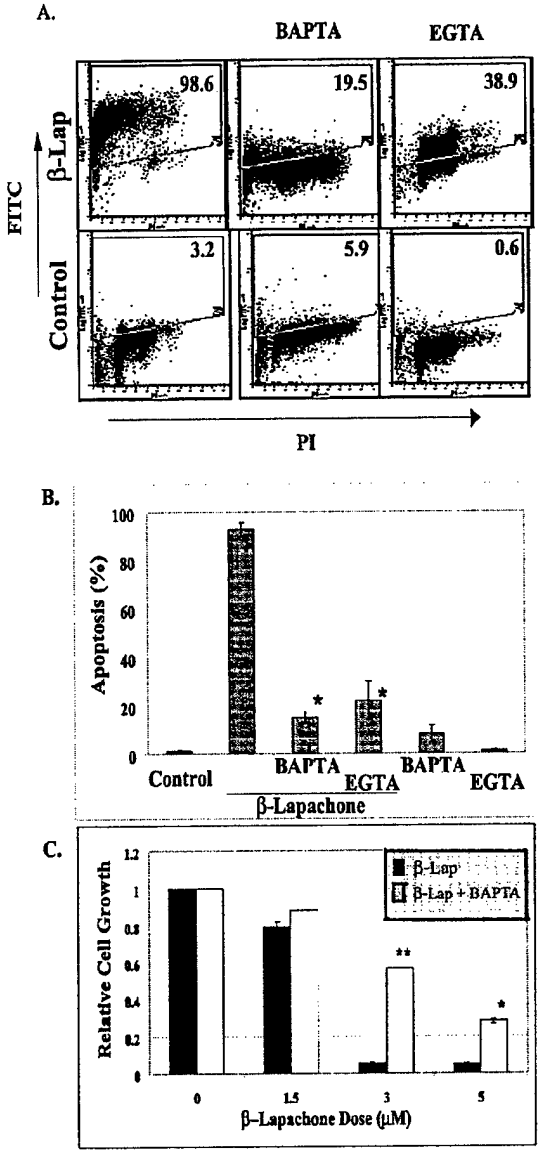

FIG. 1. **β-Lap-mediated apoptosis and relative cell growth is Ca^{2+}-dependent.** DNA fragmentation was assessed using the TUNEL assay. Log phase MCF-7 cells were treated with the indicated Ca^{2+} chelator for 30 min prior to a 4-h pulse of 5 μM β-Lap. TUNEL assays were performed to monitor apoptosis 24 h after β-Lap addition (*A* and *B*). *A*, shown are the results of any one experiment from studies performed at least three times. The *number* in the *upper right* corner represents percent cells staining positive in the TUNEL assay. Results are graphically summarized in *B* as the average of at three independent experiments, mean ± S.E. Student's *t* test for paired samples, experimental group compared with MCF-7 cells treated with β-Lap alone are indicated (* $p < 0.01$). *C*, cells were exposed to a 4-h pulse of various concentrations of β-Lap either alone (*closed*), or after a 30-min pretreatment with 5 μM BAPTA-AM (*open*). Relative DNA per well was determined by Hoescht 33258 fluorescence, and graphed as relative growth (treated/control DNA); mean relative DNA per well, ± S.E. Shown are representative results of experiments performed at least twice. Student's *t* test for paired samples, experimental group compared with MCF-7 cells treated with β-Lap alone are indicated (*, $p < 0.05$; and **, $p < 0.005$).

Ca^{2+} Chelators Do Not Block Apoptosis Induced by Other Agents—It was possible based on the data in Fig. 1 that calcium chelators may block β-Lap-mediated apoptosis by sequestering

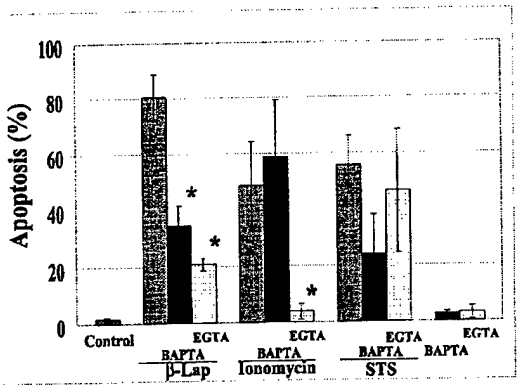

FIG. 2. **Ca^{2+} chelators did not block Ca^{2+}-activated endonuclease activation after β-Lap.** NQO1-expressing MDA-468-NQ3 cells (generated from non-expressing human breast cancer cells (5)) were treated with either 3 mM EGTA or 30 μM BAPTA-AM for 30 min prior to drug addition; either a 4-h pulse of 8 μM β-Lap, or 24 h continuous treatment of 10 μM ionomycin or 1 μM STS. Cells were then analyzed using the TUNEL assay for DNA fragmentation. Shown are mean ± S.E. of at least two independent experiments. Student's *t* test for paired samples, experimental group compared with cells treated with drug alone are indicated (*, $p < 0.05$).

calcium required for the activation of apoptotic endonucleases. We, therefore, examined both intra- and extracellular Ca^{2+} chelators for their ability to prevent apoptosis in NQO1-transfected MDA-468 (MDA-468-NQ3) cells induced by β-Lap, ionomycin (which induces Ca^{2+}-mediated cell death (36)), and staurosporine (STS, which inhibits protein kinase C and works via a caspase-mediated cell death pathway (43, 44)). We used MDA-468-NQ3 cells to assay for caspase-mediated endonuclease activation and DNA fragmentation since they express the endonuclease-activating caspase 3, unlike MCF-7 cells (45). We previously demonstrated that MDA-468-NQ3 cells responded similarly to β-Lap as MCF-7 cells (Fig. 2 and Ref. 5). EGTA significantly protected MDA-468-NQ3 cells against ionomycin-induced apoptosis, but not against STS-induced apoptosis (Fig. 2). MDA-468-NQ3 cells treated for 24 h with 10 μM ionomycin exhibited 49% apoptotic cells, whereas, MDA-468-NQ3 cells pretreated for 30 min with 3 mM EGTA followed by a 24-h exposure to ionomycin exhibited only 4% apoptotic cells. Cells treated for 24 h with 1 μM STS in the absence or presence of 3 mM EGTA exhibited 56 and 46% apoptosis, respectively. BAPTA-AM (10 μM) did not significantly block apoptosis induced by ionomycin. BAPTA-AM pretreatment of STS-exposed MDA-468-NQ3 cells did not significantly decrease apoptosis ($p < 0.4$) compared with cells exposed to STS alone; the modest effect of BAPTA-AM on STS-induced apoptosis may reflect the Ca^{2+} dependence of the apoptotic endonucleases involved in this response. Neither BAPTA-AM nor EGTA alone elicited apoptotic responses at the doses used in the aforementioned experiments (Figs. 1*B* and 2). Furthermore, preliminary data suggest that DFF45 (ICAD) was cleaved in NQO1-expressing MCF-7 or MDA-468-NQ3 cells at 8 h after β-Lap treatment, in a temporal manner corresponding to the induction of apoptosis (data not shown). Cleavage of DFF45, an endogenous inhibitor of the magnesium-dependent and Ca^{2+}-independent apoptotic endonuclease, DFF40 (CAD), suggests that DFF40 is activated following treatment with β-Lap. Taken together with results in Fig. 1, these data strongly suggest that a rise in intracellular Ca^{2+} levels is part of a critical signaling pathway for the induction of apoptosis in NQO1-expressing human breast cancer cells following β-Lap exposure.

Exposure of NQO1-expressing MCF-7 Cells to β-Lap Results in Increased Intracellular Ca^{2+}—We next directly examined whether intracellular Ca^{2+} levels were increased in log-phase MCF-7 cells after β-Lap treatment using the cell-permeant intracellular Ca^{2+} indicator dye, fluo-4. Cells were loaded with 5 μM fluo-4-AM, and where indicated, 20 μM BAPTA-AM, incubated for ~25 min to allow for the dye to permeate cells, rinsed, and then incubated for an additional ~20 min for hydrolysis of the AM-ester. Following drug addition, images were collected every 90 s for ~60 min using confocal microscopy. Three basal images were recorded before drug addition and average pixels per cell were determined (indicative of fluo-4 fluorescence and, therefore, basal intracellular Ca^{2+} levels) and used for analyses over time. The fluorescence of basal images were averaged and set to equal one; fold increases were determined from changes in fluo-4 fluorescence over control.

After exposure to 8 μM β-Lap, MCF-7 cells exhibited an ~2-fold increase in fluo-4 fluorescence from 4 to 9 min, after which time Ca^{2+} levels returned to basal levels in a majority of cells examined (43 of 50, 86%) (Fig. 3*A*). The rise in intracellular Ca^{2+} levels in MCF-7 cells following β-Lap exposure was prevented by preloading cells with BAPTA-AM (20 μM) (Fig. 3*B*). Interestingly, not all β-Lap-exposed MCF-7 cells were affected by pretreatment with BAPTA-AM; 3 of 26 cells (12%) exhibited a rise in intracellular Ca^{2+} levels after exposure to β-Lap despite the presence of this Ca^{2+} chelator. However, BAPTA-AM pretreated MCF-7 cells that did exhibit a rise in intracellular Ca^{2+} levels following β-Lap treatment exhibited a similar, but delayed Ca^{2+} increase (10–20 min), as compared with β-Lap-exposed MCF-7 cells in the absence of BAPTA-AM (4–9 min). This may be due to a saturation of the chelator or heterogeneity of the tumor cell population. These results are consistent with previous reports that the buffering capacity of BAPTA-AM may be overwhelmed with time (34, 46). Higher doses of BAPTA-AM were not used due to toxicity caused by the drug alone (data not shown).

Since the ER is a major store of Ca^{2+} in the cell, we tested if the initial rise in intracellular Ca^{2+} levels after exposure of MCF-7 cells to β-Lap was due to release of Ca^{2+} from this organelle. If β-Lap exposure led to release of Ca^{2+} stored in the ER, then TG (a sarcoplasmic reticulum Ca^{2+}-ATPase pump inhibitor) administration should not cause additional Ca^{2+} release. Similarly, if the sequence of drug administration were reversed, additional Ca^{2+} release would also not be observed. When β-Lap was added after TG-induced depletion of ER Ca^{2+} stores, no measurable rise in intracellular Ca^{2+} levels occurred in 25 of 27 (93%) cells analyzed (Fig. 3*C*). Similarly, when TG was added to cells after β-Lap, only 1 of 18 (6%) cells that initially responded to β-Lap exhibited a rise in intracellular Ca^{2+} levels following subsequent TG administration (Fig. 3*D*). At the end of the experiment, all cells analyzed remained responsive to ionomycin. Thus, cells exposed to β-Lap and/or TG were still capable of altering Ca^{2+} levels, and the Ca^{2+} indicator dye was not saturated. We noted that the increase in fluo-4 fluorescence (2–3-fold over basal levels, Fig. 3*A*) in MCF-7 cells observed after exposure to β-Lap was comparable to that elicited by TG (1.5–2.5-fold over basal levels, Fig. 3*C*), further suggesting that the two agents mobilized the same ER pool of Ca^{2+}. All cells analyzed started with comparable basal levels of Ca^{2+} and appeared to load equal amounts of the indicator dye, as determined by basal fluorescence (measured by pixels per cell) at the beginning of each analysis; relative basal fluo-4 fluorescence for each experiment in Fig. 3 were: A, 56 ± 7; B, 52 ± 7; C, 78 ± 8; D, 79 ± 8 S.E. Untreated or BAPTA-AM-loaded MCF-7 cells did not show any fluctuations in basal Ca^{2+} levels during the time course of the experiment, nor did any of the drugs interfere with the Ca^{2+} indicator dye (data not shown).

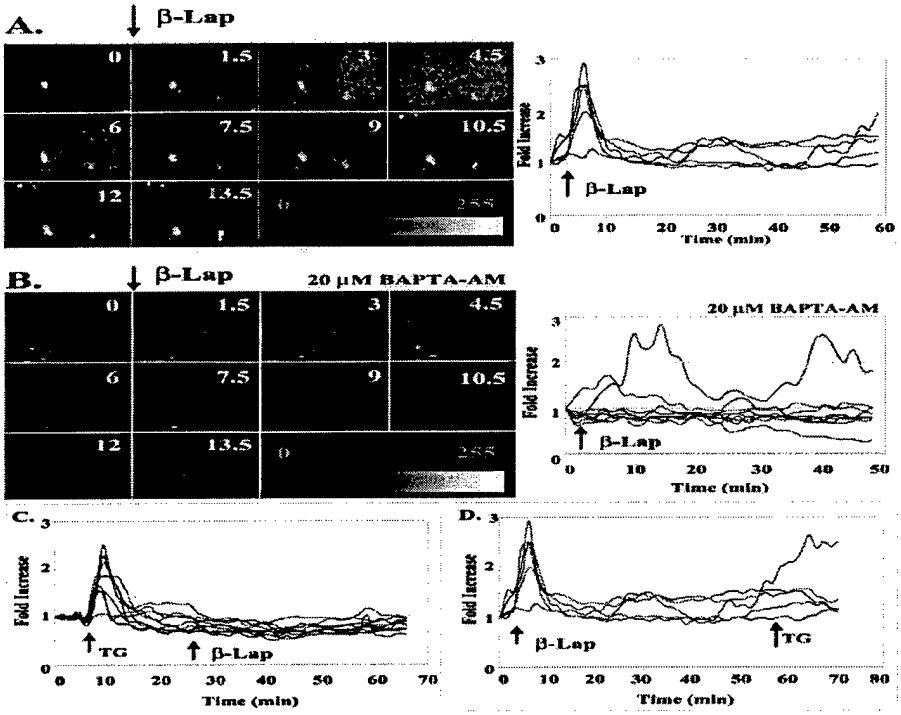

FIG. 3. **Intracellular Ca^{2+} changes after β-Lap.** Intracellular Ca^{2+} levels were measured in live cells via confocal microscopy using the Ca^{2+} indicator dye, fluo-4-AM. MCF-7 cells were loaded with either fluo-4-AM alone (*A, C,* and *D*) or fluo-4-AM and 20 μM BAPTA-AM (*B*). β-Lap (8 μM) was added to cells after basal images were recorded. Images were collected every 90 s for 45–75 min, as indicated. The *number* in the *upper right corner* of each Ca^{2+} image represents the time (min) after β-Lap addition. *A,* representative cells before and after β-Lap treatments are shown as pseudocolored images. These results are also displayed in *graph form* showing fold change (as compared with basal levels) in fluo-4 fluorescence in cells after β-Lap treatment over time, with or without co-loading of BAPTA-AM (*A* and *B*). *C,* TG (200 nM) was added to MCF-7 cells after basal images were recorded. Once fluo-4 fluorescence returned to basal levels, cells were subsequently exposed to β-Lap. *D,* β-Lap was added to MCF-7 cells after basal images were recorded. After fluo-4 fluorescence returned to basal levels, TG was subsequently added to the cells. Each *line* represents the change in fluo-4 fluorescent emission of an individual cell over time; each graph is representative of one of at least three independent experiments.

Loss of Mitochondrial Membrane Potential After β-Lap Is Attenuated by Intracellular, but Not Extracellular, Ca^{2+} Chelation—Mitochondrial membrane potential was previously shown to drop from a hyperpolarized state to a depolarized state after treatment of various human cancer cells with β-Lap (41). A drop in mitochondrial membrane potential in β-Lap-treated cells was accompanied by a concomitant release of cytochrome *c* into the cytosol (41). To explore whether early changes in intracellular Ca^{2+} levels were upstream of mitochondrial changes in NQO1-expressing breast cancer cells, log phase MCF-7 cells were pretreated for 30 min with either 10 μM BAPTA-AM or 3 mM EGTA and then exposed to 5 μM β-Lap for 4 h. Prior to analyses, cells were loaded with JC-1, a cationic dye commonly used to monitor alterations in mitochondrial membrane potential (47, 48). Mitochondrial depolarization measurements using JC-1 were indicated by a decrease in the red/green fluorescence intensity ratio (a movement of events from *upper left* to *lower right*, Fig. 4), as seen following a 10-min treatment with the potassium ionophore, valinomycin (100 nM), which causes a collapse of mitochondrial membrane potential by uncoupling mitochondrial respiration (Fig. 4*e*) (49); cells in the *upper left-hand* quadrant exhibited high mitochondrial membrane potential, whereas, cells in the *lower right-hand* quadrant have low mitochondrial membrane potential and are depolarized. Cells in the *upper right-hand* quadrant exhibited intermediate membrane potential. Mitochondrial membrane potential decreased in MCF-7 cells in a time- and dose-dependent manner following exposure to β-Lap (Figs. 4, *a-d*, and data not shown). By 4 h, the majority of β-Lap-treated MCF-7 cells exhibited low mitochondrial membrane potential (53%), while the majority of control cells maintained high mitochondrial membrane potential (51%) (Fig. 4, *b, a* and *g, f*, respectively). This drop in mitochondrial membrane potential observed 4 h after treatment with β-Lap (low, 53%) was abrogated by pretreatment with BAPTA-AM (low, 23%), but not by EGTA (low, 48%) (Fig. 4, *g-i*, respectively). Pretreatment with 10 μM BAPTA-AM prevented the decrease in mitochondrial membrane potential (low, 23%); however, BAPTA-AM did not maintain β-Lap-exposed cells in a high-potential state (high, 28%) as observed in control untreated cells (high, 51%). Approximately half of the BAPTA-AM-exposed cells were in an intermediate membrane potential state (45%) (Fig. 4*h*). We noted, however, that BAPTA-AM or EGTA exposures alone caused depolarization of the mitochondria, with a majority of the cells residing in the same intermediate energized state as observed following BAPTA-AM and β-Lap (Fig. 4, *j-k*). Therefore, BAPTA-AM prevented mitochondrial depolarization induced by β-Lap to the same extent as in cells treated with BAPTA-AM alone. Pretreatment with 3 mM EGTA did not affect the loss of mitochondrial membrane potential caused by β-Lap (low 48%), implying that an early rise in intracellular Ca^{2+} levels from intracellular stores was sufficient to cause a drop in mitochondrial membrane potential, and that extracellular calcium was not needed for these effects in β-Lap-treated cells (Fig. 4, *h-i*).

Loss of ATP After β-Lap Is Attenuated by Intracellular Ca^{2+} Chelation—The bioactivation of β-Lap by NQO1 is thought to lead to a futile cycling between quinone and hydroquinone forms of the compound, presumably due to the instability of the

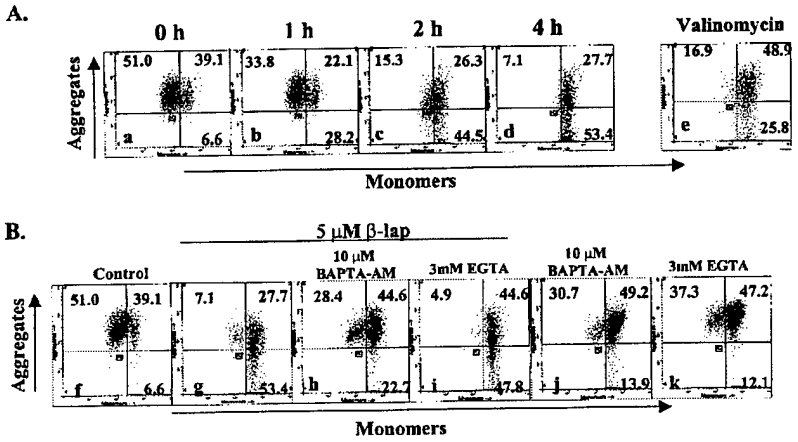

FIG. 4. **β-Lap-induced loss of mitochondrial membrane potential is mediated by alterations in Ca^{2+} homeostasis.** Mitochondrial membrane potential was measured in control or drug-treated MCF-7 cells with the JC-1 dye. *A*, cells were treated with 5 μM β-Lap and assayed for changes in mitochondrial membrane potential at 1, 2, and 4 h post-treatment. Exposure of MCF-7 cells to 100 nM valinomycin for 15 min served as a positive control as described (49). Cells in the *upper left-hand* quadrant exhibit high mitochondrial membrane potential, while cells in the *lower right-hand* quadrant exhibit low mitochondrial membrane potential. *B*, cells were treated for 30 min with either 10 μM BAPTA-AM or 3 mM EGTA prior to a 4-h treatment with 5 μM β-Lap. At 4 h, cells were harvested for analyses of changes in mitochondrial membrane potential using JC-1 as described above. Shown are representative experiments performed at least three times, and *numbers* in each quadrant represent the average of cells in that quadrant of at least three independent experiments. S.E. for any single number was not more than 11%.

hydroquinone form of β-Lap (5). This futile cycling led to depletion of NADH and NADPH, electron donors for NQO1 in *in vitro* assays (5). Exhaustion of reduced enzyme co-factors may be a critical event for the activation of the apoptotic pathway in NQO1-expressing cells following β-Lap exposure. We, therefore, measured intracellular ATP and ADP in log-phase MCF-7 cells after various doses and times of β-Lap (using a luciferase-based bioluminescent assay (42)). Intracellular ATP levels were reduced in MCF-7 cells after treatment with β-Lap in a dose- and time-dependent manner (Fig. 5*A*). At all doses of β-Lap above the LD_{50} of the drug (~2.5 μM) in MCF-7 cells (2), intracellular ATP levels were reduced by >85% at 4 h, the time at which drug was removed (Fig. 5*A*, *left*); the loss of ATP correlated well with β-Lap-induced cell death in MCF-7 cells (Fig. 1*C*). ADP levels remained relatively unchanged after various doses of β-Lap, however, the [ATP]/[ADP][P_i] ratio decreased dramatically. Intracellular ATP levels began to drop to 70% of control levels 2 h after 5 μM β-Lap exposure, the time at which β-Lap began to elicit mitochondrial membrane depolarization (Figs. 5, *A*, *right*, and 4, *c*). ATP levels continued to drop to 8% of control levels by 4 h after drug exposure (Fig. 5*A*, *right*). In contrast, ADP levels remained relatively unchanged during the course of the experiment, with an increase at 30 min (172% control levels) that returned to control levels by 1 h post-treatment. Cellular ATP levels in β-Lap-treated cells did not appear to recover to normal levels within the 6–24-h interval after drug removal (data not shown).

Loss of ATP following β-Lap was prevented by a 30-min pretreatment with an intracellular Ca^{2+} chelator, but not an extracellular Ca^{2+} chelator (Fig. 5*B*). At 4 h, pretreatment with 10 or 30 μM BAPTA-AM elicited only 58 and 43% ATP loss, respectively, compared with β-Lap alone (92% loss). The extracellular Ca^{2+} chelator, EGTA, did not significantly affect the loss of ATP, nor [ATP]/[ADP][P_i] ratio observed in MCF-7 cells after β-Lap treatment (Fig. 5*B*). Exposure of MCF-7 cells to TG (200 nM) did not elicit decreases in ATP or ADP levels 4 h after drug exposure, compared with untreated control cells.

Ca^{2+} Chelators Prevent β-Lap-induced Proteolysis—We previously showed that apoptosis in various breast cancer cell lines induced by β-Lap was unique, causing a pattern of PARP and p53 intracellular cleavage events distinct from those induced by caspase activating agents (12). After β-Lap treatment, we observed an ~60-kDa PARP cleavage fragment and specific cleavage of p53 in NQO1-expressing breast cancer cells. Furthermore, we showed that this proteolysis in β-Lap-treated cells was the result of activation of a Ca^{2+}-dependent protease with properties similar to μ-calpain (12). PARP and p53 proteolysis in β-Lap-exposed, NQO1-expressing cells was prevented by pretreatment with the extracellular Ca^{2+} chelators, EGTA and EDTA, in a dose-dependent manner (at 8 and 24 h) (Ref. 12, and data not shown). Additionally, PARP, p53, and lamin B proteolysis induced at 24 h in MCF-7 cells following β-Lap treatment were abrogated by pretreatment with 10 or 30 μM BAPTA-AM (Fig. 6). These data strongly suggest that a Ca^{2+}-dependent pathway and potentially a Ca^{2+}-dependent protease are operative in β-Lap-mediated apoptosis.

A simple explanation for the aforementioned results could be that BAPTA blocks bioactivation of β-Lap by NQO1 in a manner similar to that of dicumarol (5). However, BAPTA (free acid) did not affect the enzymatic activities of NQO1 using standard enzymatic assays (data not shown) (5). The free acid (active) form of BAPTA, instead of its −AM ester form, was used in these assays since intracellular accumulation of this Ca^{2+} chelator was not necessary and was physiologically relevant in the *in vitro* enzyme assay. Using β-Lap as a substrate, NQO1 enzymatic activity in the presence of 10 mM BAPTA (a dose of the free acid form of BAPTA that was >1000-fold higher than that used in the experiments of Figs. 1–6) was reduced by <20%. Thus, BAPTA-AM did not affect the activity of NQO1, a two-electron reductase required for β-Lap cytotoxicity (5). We conclude that BAPTA-AM prevents β-Lap-induced apoptosis by blocking Ca^{2+}-mediated signaling events via chelating intracellular Ca^{2+}.

β-Lap Bioactivation by NQO1 Is Critical for Ca^{2+}-mediated Signaling—We previously reported that cells expressing NQO1 are more sensitive to the cytotoxic effects of β-Lap (5).[2] NQO1 is inhibited by dicumarol, which competes with NADH or NADPH for binding to the oxidized form of the enzyme. Dicumarol thereby prevents reduction of quinones (50, 51). We demonstrated that dicumarol attenuates β-Lap-mediated proteolysis of apoptotic substrates (*e.g.* PARP and p53), apoptosis, and survival in NQO1-expressing cells (5).[2] As expected, increases

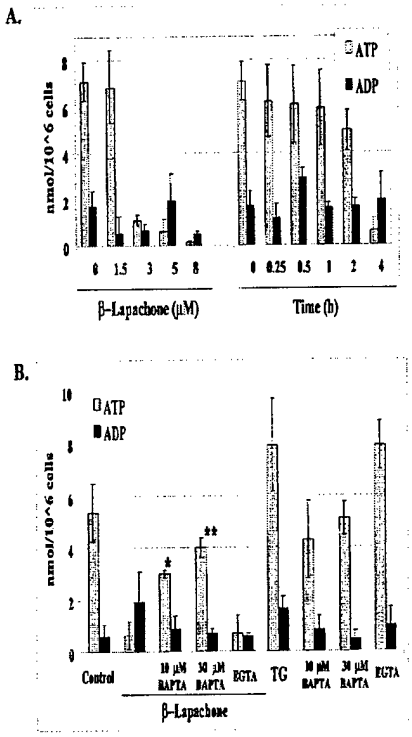

FIG. 5. **ATP depletion after β-Lap treatment is Ca^{2+} dependent.** Intracellular ATP and ADP levels were measured using a luciferase-based bioluminescent assay. *A*, cells were treated with the indicated dose of β-Lap for 4 h or were treated with 5 μM β-Lap for the time indicated, and harvested for ATP analyses. ATP levels were expressed as nanomoles of ATP per 10^6 cells. Purified ATP was used as a standard to determine intracellular ATP concentrations. *B*, cells were either pretreated or untreated with the indicated Ca^{2+} chelators for 30 min prior to drug addition, and β-Lap (5 μM) was then added for 4 h. Cells were harvested for analyses following β-Lap exposure. Results represent the average of at least three independent experiments, ± S.E. Student's *t* test for paired samples, experimental group compared with drug alone are indicated (*, $p < 0.05$; **, $p < 0.01$).

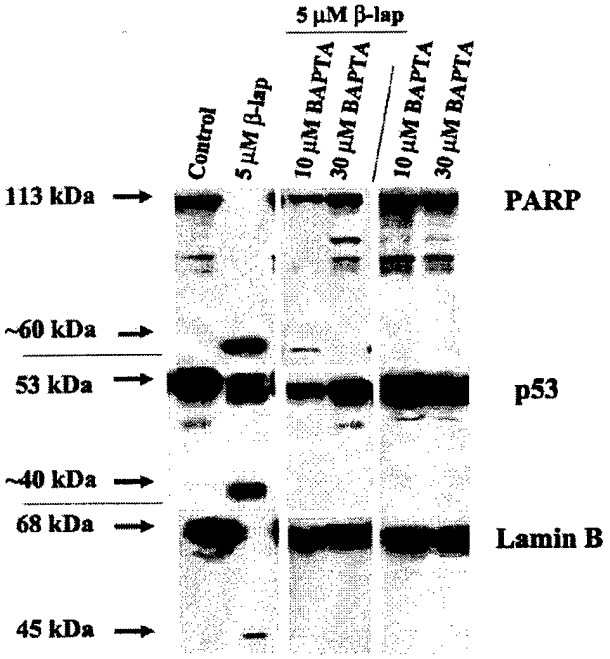

FIG. 6. **Intracellular Ca^{2+} chelators prevent apoptotic proteolysis after β-Lap treatment.** Apoptotic proteolysis was measured in MCF-7 cells exposed to a 4-h pulse of 5 μM β-Lap, with or without a 30-min pretreatment of the indicated dose of BAPTA-AM. Whole cell extracts were prepared 24 h after drug addition, and analyzed using standard Western blotting techniques with antibodies to PARP, p53, and lamin B. Shown is a representative Western blot of whole cell extracts from experiments performed at least three times.

in intracellular Ca^{2+} levels in NQO1-expressing human cancer cells elicited by β-Lap were abrogated by co-treatment with 50 μM dicumarol in 26 of 27 cells (96%) examined (Fig. 7*A, lower panel*). The ability of dicumarol to inhibit increases in intracellular Ca^{2+} levels was greater than that observed with BAPTA-AM, where intracellular Ca^{2+} level increases were prevented in only 89% of cells examined (Fig. 3*B*). Thus, NQO1 was critical for the rise in intracellular Ca^{2+} levels observed in MCF-7 cells after β-Lap exposure.

Mitochondrial membrane depolarization induced by β-Lap was also abrogated by pretreatment with dicumarol (Fig. 7*B*). By 4 h, the majority of β-Lap-treated cells exhibited low mitochondrial membrane potential (58%), while very few control cells were depolarized (9%) (Fig. 7*B*). Pretreatment with dicumarol attenuated this response to β-Lap, with only 34% being depolarized. The inability of dicumarol to prevent mitochondrial depolarization in 34% of β-Lap-treated cells was probably due to the high background of control cells (20%) that were depolarized after exposure to dicumarol alone. In comparison with intracellular Ca^{2+} buffering, BAPTA-AM elicited only a minor depolarization of the mitochondria on its own (low, 14%) and thus was able to elicit a greater protective effect (Fig. 4*B*); only 23% of cells exposed to BAPTA-AM and β-Lap exhibited low mitochondrial membrane potential as compared with β-Lap exposed cells in the presence of dicumarol (34%).

The dramatic loss of intracellular ATP in MCF-7 cells following β-Lap exposure was inhibited by a 30-min pretreatment with 50 μM dicumarol (Fig. 7*C*). β-Lap-treated MCF-7 cells pretreated with dicumarol exhibited only 34% loss of intracellular ATP, compared with 92% loss after β-Lap treatment alone (Fig. 7*C*). ADP levels were not altered by any of the treatments used, however, the [ATP]/[ADP][P_i] ratio decreased dramatically in β-Lap-treated cells, and was only partially decreased with dicumarol pretreatment alone, as compared with control untreated cells.

Dicumarol also abrogated DNA fragmentation induced by β-Lap in MCF-7 cells. MCF-7 cells exhibited 94% apoptosis following β-Lap exposure that was prevented by a 30-min pretreatment with 50 μM dicumarol; only 6% of the cells staining positive in a TUNEL assay at 24 h post-treatment (Fig. 7*D*). These data are consistent with prior results (5), and correlate well with the survival protection afforded by dicumarol to β-Lap-treated cells. Dicumarol did not induce DNA fragmentation on its own. These data are consistent with the protection from apoptosis observed with either intra- and extracellular Ca^{2+} chelators. BAPTA-AM or EGTA protected β-Lap exposed MCF-7 cells from apoptosis (Fig. 1, *A* and *B*). Collectively, these data implicate the bioactivation of β-Lap by NQO1 as a critical step in the rise of intracellular Ca^{2+} levels following β-Lap exposure, and thus β-Lap-mediated downstream apoptotic events.

DISCUSSION

When homeostatic mechanisms for regulating cellular Ca^{2+} are compromised, cells may die, either by necrosis or apoptosis (20, 21, 36). We demonstrated that bioactivation of β-Lap by NQO1 induced cell death in a manner that was dependent upon Ca^{2+} signaling (Figs. 1–6). β-Lap can be reduced by NQO1 and

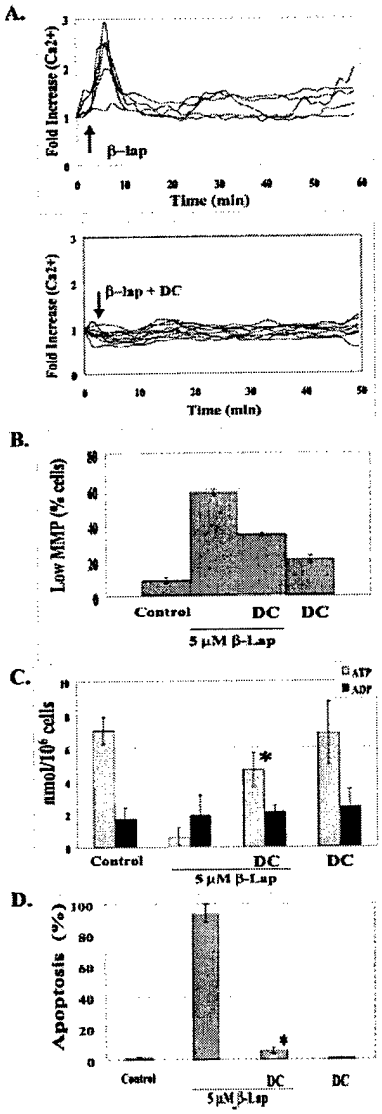

FIG. 7. **NQO1-dependent activation of β-Lap is critical for Ca^{2+} signaling.** *A*, intracellular Ca^{2+} was measured on live cells using the Ca^{2+} indicator dye, fluo-4-AM, and confocal microscopy as described in the legend to Fig. 3. Three basal images were recorded before drug treatments. β-Lap (8 μM) was then added to MCF-7 cells, either alone (*upper panel*) or in combination with 50 μM dicumarol (*lower panel*). Images were collected every 90 s for 50–60 min. Shown are representative graphs displaying changes in fluo-4 fluorescence for the duration of the experiment. Each *line* represents the fold change in fluo-4 fluorescent emission (as compared with basal levels) of an individual cell from one experiment, and the graph is representative of experiments performed at least three times. *B*, mitochondrial membrane potential was measured using the JC-1 dye as described in the legend to Fig. 4. MCF-7 cells were treated with 50 μM dicumarol 30 min prior to β-Lap exposure. Four hours later, cells were harvested for analyses of mitochondrial membrane potential. Shown are mean ± S.E. of the percentage of cells with low mitochondrial membrane potential of at least two independent experiments. *C*, ATP and ADP levels were assayed as described in the legend to Fig. 5. Cells were pretreated with dicumarol for 30 min prior to drug addition, 5 μM β-Lap was added for 4 h, and cells were harvested immediately thereafter for analyses. Results represent the mean of at least three independent experiments ± S.E. Student's *t* test for paired samples, experimental groups compared with drug alone are indicated (* $p < 0.05$). *D*, apoptosis, using the TUNEL assay, was assessed as per Fig. 1. MCF-7 cells were treated with 50 μM dicumarol 30 min prior to a 4-h exposure of 5 μM β-Lap. Cells were then harvested for TUNEL analyses at 24 h post-treatment. Shown are mean ± S.E. of at least three independent experiments. Student's *t* test for paired samples, experimental groups compared with β-Lap exposure alone are indicated (*, $p < 0.005$). *DC*, 50 μM dicumarol.

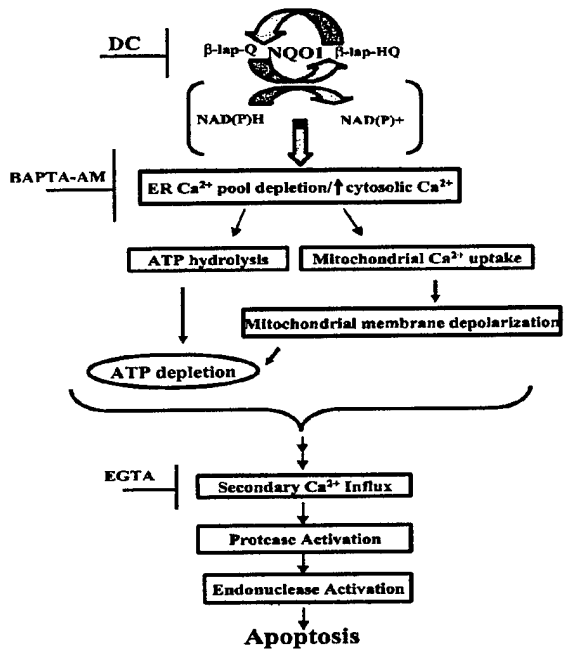

FIG. 8. **Proposed model for β-lapachone-mediated apoptosis in NQO1-expressing cells.** In cells that express NQO1, β-Lap is reduced from the quinone (*β-lap-Q*) to the hydroquinone (*β-lap-HQ*) form in a futile cycle that results in dramatic losses of NAD(P)H (5). During the metabolism of β-Lap by NQO1, Ca^{2+} is subsequently released from the ER causing a rise in cytosolic Ca^{2+} levels by an as yet unknown mechanism. To maintain low cytoplasmic Ca^{2+} levels, we theorize that mitochondria sequester Ca^{2+} and numerous cellular ATPases probably function to pump Ca^{2+} out of the cytosol. This leads to mitochondrial membrane depolarization and ATP hydrolysis, respectively (Figs. 4 and 5). Sustained depolarization of the mitochondrial membrane leads to further loss of ATP and prevents ATP synthesis by inhibiting respiration. The loss of ATP disrupts ionic homeostasis within the cell and thereby allows extracellular Ca^{2+} to enter the cell down its concentration gradient (see "Discussion"). The secondary rise in cytosolic Ca^{2+} levels leads to protease (presumably activation of calpain or a calpain-like protease) and, thus, endonuclease (DFF40) activation, ultimately resulting in apoptosis.

may undergo futile cycling between quinone and hydroquinone forms (β-Lap-Q and β-Lap-HQ, Fig. 8), presumably depleting NADH and/or NADPH in the cell (5). We theorize that depletion of NAD(P)H, along with a rise in intracellular Ca^{2+} levels in response to β-Lap, activate a novel caspase-independent apoptotic pathway, as described in this paper and previously (2, 5, 12). The rise in intracellular Ca^{2+} appears to be dependent upon the bioactivation of β-Lap by NQO1, suggesting a critical and necessary signaling role for Ca^{2+} in the downstream apoptotic pathway induced by this drug. Dicumarol completely abrogated intracellular Ca^{2+} changes (Fig. 7), as well as apoptosis and survival, following β-Lap exposure of NQO1-expressing cells (5).[2] When increases in intracellular Ca^{2+} levels were directly prevented by pretreatment with BAPTA-AM, downstream apoptotic responses, as well as lethality, caused by β-Lap were prevented; when corrected for BAPTA-AM affects alone, β-Lap-induced apoptosis, proteolysis, and lethality were essentially blocked by preventing early Ca^{2+} release from ER stores. Thus, correcting for the BAPTA-AM affects alone, the role of Ca^{2+} in β-Lap-mediated apoptosis may be more significant that that revealed by the data shown. These data strongly

suggest that DNA fragmentation, mitochondrial membrane depolarization, ATP loss, and apoptotic proteolysis were a consequence of the increase in intracellular Ca^{2+} levels (Figs. 1–6 and 8). Interestingly, the cell death pathway induced by β-Lap was quite distinct from that observed after exposure to TG, an agent known to specifically cause release of Ca^{2+} from ER stores and mediate caspase-dependent apoptosis (24, 28, 33, 52). Thus, Ca^{2+} release was necessary for β-Lap-induced cytotoxicity, but apparently not sufficient for the unique apoptotic responses induced by β-Lap.

β-Lap and TG-induced Similar Ca^{2+} Responses, but Different Patterns of Apoptosis—β-Lap elicited an early rise in intracellular Ca^{2+} levels from the same ER store as released by TG, however, subsequent cell death processes were remarkably different between the two compounds. TG is known to cause transient increases in intracellular Ca^{2+} levels, however, these were insufficient to induce apoptosis. Much like β-Lap, Ca^{2+} was needed from the extracellular milieu, along with a sustained increase in intracellular Ca^{2+} levels, for TG-induced apoptosis (23) in MCF-7 cells (27). Depolarization of the mitochondrial membrane potential and loss of intracellular ATP in cells exposed to β-Lap, may have prevented plasma membrane Ca^{2+} pumps and ER Ca^{2+} pumps from functioning and maintaining Ca^{2+} homeostasis. This, in turn, may have facilitated Ca^{2+} leakage down its concentration gradient into the cytosol, providing a secondary and sustained elevation of Ca^{2+} that initiated a protease cascade(s) and ultimately caused apoptosis after exposure to β-Lap. This is consistent with what we observed in NQO1-expressing cells after β-Lap treatment and co-administration of Ca^{2+} chelators. Buffering intracellular Ca^{2+} with BAPTA-AM partially abrogated all of the downstream events induced in MCF-7 cells by β-Lap (and thus prevented secondary Ca^{2+} entry by buffering the initial rise in cytosolic Ca^{2+}). In contrast, extracellular chelation by EGTA only prevented those events initiated by secondary Ca^{2+} entry (*e.g.* protease activation and DNA fragmentation). Thus, a secondary rise in intracellular Ca^{2+} levels after exposure to β-Lap seems probable, and necessary, for protease activation and DNA fragmentation as was observed for TG-induced caspase-mediated apoptosis (23, 27). However, a secondary influx of Ca^{2+} does not appear to be necessary for reduction in mitochondrial membrane potential or loss of intracellular ATP after β-Lap exposure, since EGTA did not prevent these responses.

Although MCF-7 cells treated with β-Lap had similar calcium responses, as do TG-exposed cells, β-Lap-exposed cells exhibited a very different pattern of apoptosis than TG-treated cells. β-Lap-exposed cells exhibit loss of intracellular ATP and a decrease in the [ATP]/[ADP][P_i] ratio. In contrast, TG-exposed cells did not exhibit loss of ATP (Fig. 5, and as reported by Ref. 53). Our data suggest that in contrast to TG where ATP-dependent caspase activation results in cell death (28, 33, 34, 54), an ATP-independent protease is activated after exposure to β-Lap. Ca^{2+} may regulate apoptosis by activating Ca^{2+}-dependent protein kinases and/or phosphatases leading to alterations in gene transcription. However, with the rapid loss of intracellular ATP after exposure to β-Lap (2–4 h, Fig. 5), β-Lap-mediated cell death unlikely involves stimulated kinases or phosphatases or new protein synthesis. Instead, indirect kinase inhibition, due to ATP depletion, along with continued phosphatase activity is likely. Consistent with this notion, we found dramatic de-phosphorylation of pRb in cells exposed to β-Lap at 3 h (2), a time consistent with loss of ATP following exposure to this drug. Furthermore, loss of ATP at 2 h may also be responsible for inhibition of NF-κB activation induced by tumor necrosis factor-α in β-Lap pre-exposed cells (55), since significant loss of ATP would prevent proteosome-mediated IκB degradation. Thus, Ca^{2+}-dependent loss of ATP in NQO1-expressing cells following β-Lap treatment may explain the reported pleiotropic effects of this agent.

β-Lap-exposed cells also exhibited a very different pattern of substrate proteolysis compared with that observed after TG (2, 12, 28). We previously showed that β-Lap elicited a unique cleavage of PARP (~60-kDa fragment), compared with the classical caspase-3-mediated fragmentation of the protein (~89 kDa) observed after TG exposure (data not shown and Ref. 28). In a variety of NQO1-expressing cells exposed to β-Lap, atypical PARP cleavage was inhibited by the global cysteine protease inhibitors, iodoacetamide and *N*-ethylmaleimide, as well as the extracellular Ca^{2+} chelators, EGTA and EDTA (12). In addition, β-Lap-mediated apoptotic responses were insensitive to inhibitors of caspases, granzyme B, cathepsins B and L, trypsin, and chymotrypsin-like proteases (12). In contrast, classic caspase inhibitors blocked TG-induced caspase activation and apoptosis (28). Caspase activation, as measured by procaspase cleavage via Western blot analyses, does not occur following β-Lap exposures.[3] Thus, protease activation after β-Lap treatment appears to be Ca^{2+}-dependent, or alternatively, is activated by another protease or event that is Ca^{2+}-dependent (Figs. 1–6 and Ref. 12).

Loss of Reducing Equivalents Is Also Necessary for β-Lap-mediated Apoptosis, Similar to Menadione-mediated Apoptosis—Menadione is a quinone that can be detoxified by NQO1 two-electron reduction. However, menadione can also be reduced through two, one-electron reductions via other cellular reductases (56), thus eliciting menadione's toxic effects. Menadione toxicity, elicited via two, one-electron reductions, exhibited many similarities to β-Lap-mediated, NQO1-dependent, toxicity (5). These included: (*a*) elevations in cytosolic Ca^{2+} (57, 58); (*b*) NAD(P)H depletion (5, 59, 60); (*c*) ATP depletion (<0.1% control)[3] (61–63); and (*d*) mitochondrial membrane potential depolarization[3] (64). We previously demonstrated that menadione caused similar substrate proteolysis (p53 and atypical PARP cleavage) in NQO1-deficient cells, or at high doses in cells that express NQO1 where detoxification processes were over-ridden (5).[3] The semiquinone form of menadione can undergo spontaneous oxidation to the parent quinone (59, 63, 65, 66); a pattern similar to the futile cycling observed after β-Lap bioactivation by NQO1 (5). Loss of reducing equivalents, such as NADH, due to the futile cycling of menadione may cause inactivation of the electron transport chain with the concomitant loss of mitochondrial membrane potential, and thus, loss of ATP (67, 68). These responses were also observed in MCF-7 cells exposed to β-Lap (Figs. 4 and 5). Extensive mitochondrial Ca^{2+} accumulation can also mediate mitochondrial depolarization (69, 70). Thus, Ca^{2+} sequestration may elicit mitochondrial membrane depolarization and consequent ATP depletion in cells exposed to β−lap. These data further suggest that Ca^{2+} is necessary for β-Lap-mediated cell death, but other factors are apparently needed for the initiation of the novel execution apoptotic pathway observed in cells treated with this compound.

The rise in intracellular Ca^{2+} appears to be dependent on the bioactivation of β-Lap by NQO1, suggesting a critical and necessary signaling role for Ca^{2+} in the downstream apoptotic pathway induced by this drug. These data suggest that DNA fragmentation, mitochondrial membrane depolarization, ATP loss, and apoptotic proteolysis were a consequence of the increase in intracellular Ca^{2+} levels. Work in our laboratory is focused on elucidating the signaling response(s) that elicits ER Ca^{2+} release following β-Lap bioactivation by NQO1. The cell

[3] C. Tagliarino, J. J. Pink, and D. A. Boothman, unpublished results.

death pathway induced by β-Lap is quite distinct from that observed after exposure to TG, and β-Lap-mediated apoptosis exhibited many similarities to menadione-mediated apoptosis. These observations further suggest that early release of Ca^{2+} from ER stores, as well as influx of Ca^{2+} from the extracellular milieu are necessary, but not sufficient for the novel apoptotic execution pathway induced by β-Lap. Thus, changes in Ca^{2+} homeostasis in conjunction with the presumed loss of reducing equivalents are both necessary and sufficient for β-Lap-mediated apoptosis. We propose that development of β-Lap for treatment of human cancers that have elevated NQO1 levels (*e.g.* breast and lung) is warranted (6). Since most clinical agents used to date kill cells by caspase-dependent and p53-dependent pathways, and many cancers evade death by altering these pathways, development of agents that kill by specific targets (NQO1-mediated) and in p53- and caspase-independent manners are needed.

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Calpains and Apoptosis

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Calpains are a family of cysteine proteases existing primarily in two forms designated by the Ca^{2+} concentration needed for activation *in vitro*, μ-calpain (calpain-I) and m-calpain (calpain-II). The physiological roles of calpains remain unclear. Many groups have proposed a role for calpains in apoptosis, but their patterns of activation are not well characterized. Calpains have been implicated in neutrophil apoptosis, glucocorticoid-induced thymocyte apoptosis, as well as many other apoptotic pathways. Calpain activation in apoptosis is usually linked upstream or downstream to caspase activation, or in a parallel pathway alongside caspase activation. Calpains have been suggested to be involved in DNA fragmentation (via endonuclease activation), but also as effector proteases that cleave cellular proteins involved in DNA repair, membrane associated proteins and other homeostatic regulatory proteins. Recently, our laboratory demonstrated μ-calpain activation in NAD(P)H: quinone oxidoreducatse 1 (NQO1)-expressing cells after exposure to β-lapachone, a novel quinone and potential chemo- and radio-therapeutic agent. Increased cytosolic Ca^{2+} in NQO1-expressing cells after β-lapachone exposures were shown to lead to μ-calpain activation. In turn, μ-calpain activation was important for substrate proteolysis and DNA fragmentation associated with apoptosis. Upon activation, μ-calpain translocated to the nucleus where it could proteolytically cleave PARP and p53. We provided evidence that β-lapachone-induced, μ-calpain stimulated, apoptosis did not involve any of the known caspases; known apoptotic caspases were not activated after β-lapachone treatment of NQO1-expressing cells, nor did caspase inhibitors have any effect on β-lapachone-induced cell death. Elucidation of processes by which β-lapachone-stimulated μ-calpain activation and calpains ability to activate endonucleases and induce apoptosis independent of caspase activity will be needed to further develop/modulate β-lapachone for treatment of human cancers that over-express NQO1.

Cell Death

There is increasing evidence that classical apoptosis and necrosis represent only the extreme ends of a wide range of possible morphological and biochemical deaths. Cytotoxic agents kill cells with similar features to both. These mechanisms are characterized by cellular morphology and an organism's biological response after cellular death has occurred. In necrotic cell death, dying cells essentially burst, spilling their internal contents into the intercellular space, resulting in an inflammatory response and further cellular damage (Bellamy et al., 1995). Apoptosis is a genetically programmed form of cell death with distinct signaling pathways. Programmed cell death (PCD, non-agent-induced apoptosis) is involved in development and normal turnover of cells (Meier et al., 2000). Similarly, apoptosis can be triggered by cellular insults resulting in a stimulation of PCD, but often times skipping a portion of PCD's genetic control (Kerr et al., 1972; Stepczynska et al., 2001). Once apoptosis is initiated, specific proteases (e.g., caspases and/or calpains) are activated resulting in biochemical and morphological changes in the cell. These changes include: DNA cleavage (TUNEL positive cell formation), chromatin condensation, nuclear fragmentation, cleavage of apoptotic substrates (e.g., PARP, lamin B), cytoplasmic membrane blebbing and formation of apoptotic bodies (small blebs of the membrane and cytoplasm) (Gerschenson and Rotello, 1992; Patel et al., 1996). Apoptotic bodies are subsequently phagocytosed by macrophages or neighboring cells without eliciting an inflammatory response, as opposed to cells that undergo necrotic cell death (Gerschenson and Rotello, 1992).

Apoptosis is a highly regulated and active process

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that requires the participation of endogenous cellular enzymes that systemically dismantle the cell. The most well characterized proteases in apoptosis are caspases, aspartate-specific cysteine proteases. However, other proteases are implicated in apoptosis as well (e.g., calpains). Apoptosis can be divided into two phases: initiation and execution. The induction of apoptosis via the caspases leads to a downstream signaling pathway that begins with the initiator caspases (e.g., caspases 8 and 9), depending upon the triggering agent. The initiation phases of apoptosis are not entirely understood, but are clearly agent-specific. These initiator caspases, in turn, activate downstream effector caspases (e.g., caspases 3, 6, and 7) (Kuida et al., 1998; Stennicke et al., 1998). Caspases exist as zymogens, inactive proenzymes that are proteolytically cleaved to form active tetramers (reviewed in (Nicholson, 1999). The effector caspases systemically dismantle the cell by cleaving proteins that are important in DNA repair (e.g., PARP, ATM and DNA-PKcs) (Kaufmann, 1989; Lazebnik et al., 1994; Casiano et al., 1996), cellular integrity (e.g., lamins, gelsolin and fodrin)(Vanags et al., 1996; Kothakota et al., 1997; Janicke et al., 1998) and cellular homeostasis (e.g., pRb)(Janicke et al., 1996), as well as by activating other apoptotic proteases (e.g., calpains) (Wang et al., 1998; Wood and Newcomb, 1999; Kato et al., 2000), that ultimately results in apoptotic cell death.

Calpains

Other proteases have been implicated in apoptosis, but their exact mechanisms of activation and intracellular targets are not well known. One of these proteases is the Ca^{2+}-activated neutral protease, calpain (EC 3.4.22.2). Calpains are a family of Ca^{2+}-dependent cysteine proteases that catalyze proteolysis of a limited number of proteins involved in cytoskeletal remodeling and signal transduction. Calpains are also implicated in a number of physiological and pathophysiological processes. These processes include cell cycle regulation, apoptosis, necrosis, muscular dystrophies, cataractogenesis, Alzheimer's and Parkinson's diseases (Goll et al., 1992; Nixon et al., 1994; Johnson and Guttmann, 1997; Sorimachi et al., 1997; Carafoli & Molinari, 1998; Richard et al., 1999).

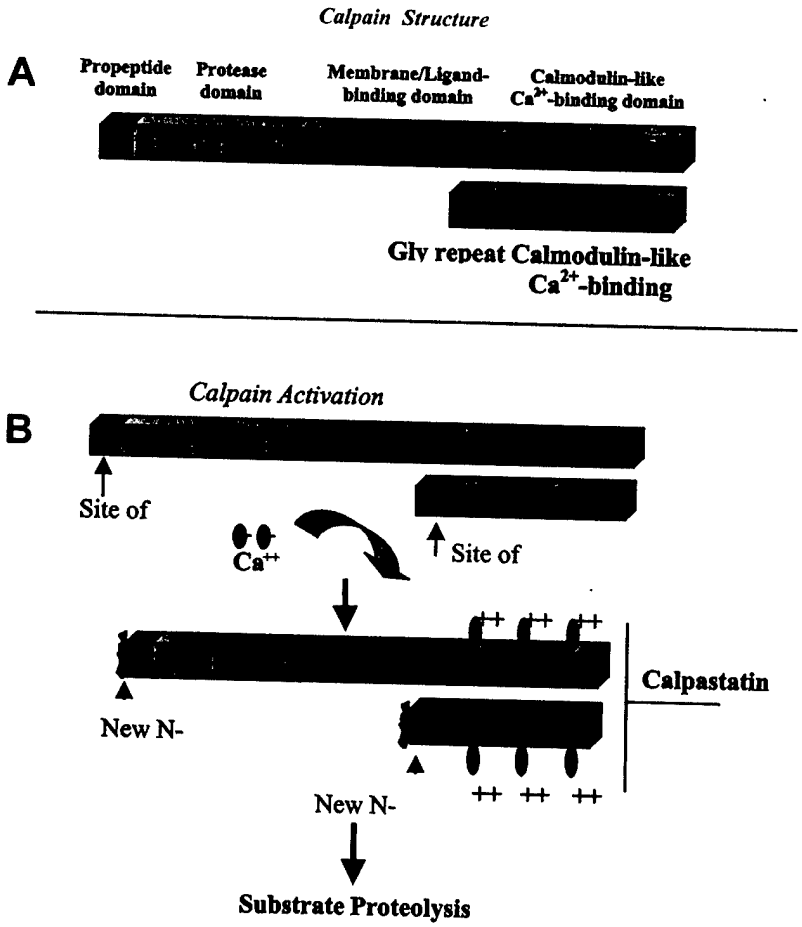

Fig. 1. A schematic diagram of calpain activation. See text for discussion.

Calpains exist primarily in two forms, designated by the Ca^{2+} concentration needed for activation *in vitro*: μ-calpain (calpain-I) and m-calpain (calpain-II). Each isoform is a heterodimer consisting of a large catalytic and a small regulatory subunit, both of which are sensitive to changes in Ca^{2+} homeostasis (Fig. 1). μ- and m-Calpains are ubiquitously expressed and predominantly located in the cytoplasm (Yoshimura et al., 1984; Banik et al., 1991), but can translocate to cellular membranes where they appear to become activated (Kawasaki and Kawashima, 1996), or to the nucleus during specific apoptotic insults (Tagliarino, et al. unpublished data).

Calpastatin, an inhibitor of μ- and m- calpains

Calpastatin is a specific endogenous inhibitor of m- and μ-calpains that binds calpains in the presence of Ca^{2+} (Fig. 2) (Cottin et al., 1981; Crawford et al., 1993). Calpastatin inhibits μ- or m-calpain in a substrate-competitive manner, and also inhibits calpains association with membranes (Maki et al., 1988; Kawasaki et al., 1989). Binding of calpains to calpastatin is reversible and does not result in any lingering loss of calpain activity (Kapprell and Goll, 1989).

Calpains have two distinct sites for binding to calpastatin, one at the active site and another at the EF-hand domain (Kawasaki and Kawashima, 1996). It is believed that calpain interacts with substrates through these same two sites.

Calpains in apoptosis

The physiological roles of calpains remain unclear (Carafoli and Molinari, 1998; Ono et al., 1998). The presence and conservation of m- and μ- calpains in almost all mammalian cells suggest that these enzymes are essential, however, the absence of specific calpain inhibitors has thus far prevented unambiguous proof of a particular physiological role. Many of the calpain inhibitors used in earlier work have been shown to also inhibit the proteosome, cathepsins, other cysteine proteases, or inhibit entirely different enzymes, for example, a protein tyrosine phosphatase (Barrett et al., 1982; Schoenwaelder and Burridge, 1999; Tenev et al., 2001). The calpain genes have no major regulatory features in their promoter regions and are usually considered to be housekeeping enzymes. The relative levels of m- and μ- calpains and of their inhibitor, calpastatin, vary from tissue to tissue, again suggesting some degree of regulation and importance in the cell (Thompson and Goll, 2000).

Calpains were first implicated in N-methyl-D-aspartate (NMDA)- and ischemia-induced apoptosis in 1993 (Roberts-Lewis et al., 1993). Since then, many other groups have proposed a role for calpains in apoptosis, but their patterns of activation are not well characterized; calpains have been implicated in neutrophil apoptosis, glucocorticoid-induced thymocyte apoptosis,

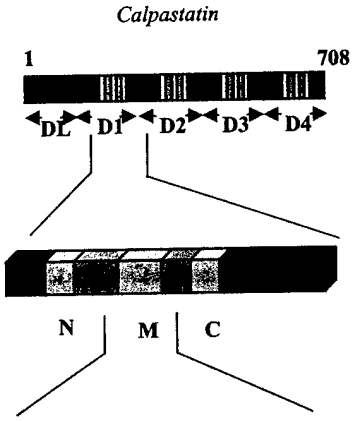

Fig. 2. A schematic diagram of calpastatin and its inhibitory domains. See text for discussion.

as well as many other apoptotic pathways (Squier and Cohen, 1997; Patel and Lane, 1999; Squier et al., 1999). Calpain activation in apoptosis is usually linked upstream or downstream to caspase activation, or in a parallel pathway alongside caspase activation (Wang et al., 1998; Wood et al., 1998; Ruiz-Vela et al., 1999; Wood and Newcomb, 1999). Caspases have been reported to be upstream of calpain activation; caspases (3 or 7) cleaved the endogenous inhibitor of calpain, calpastatin, thus allowing for increased calpain activation after apoptotic induction by anti-Fas or staurosporine exposures (Wang et al., 1998; Kato et al., 2000). Others have reported calpain activation upstream of caspase activation, demonstrating that calpains specifically trigger activation and processing of caspase 7 or 12 in various model systems (Ruiz-Vela et al., 1999; Nakagawa and Yuan, 2000). In addition, calpain may be responsible for cleaving the loop region in Bcl-xL and, therefore, turning the anti-apoptotic Bcl-xL protein into a pro-apoptotic molecule that further promotes caspase activation (Nakagawa and Yuan, 2000). Calpains can also inactivate caspases (3, 7, 8, or 9) by cleavage of the pro-caspase form to proteolytically inactive fragments (McGinnis et al., 1999; Chua et al., 2000). In addition, calpain activation in apoptosis can occur concurrently with, or in the absence of, caspase activation (Nath et al., 1996b; McGinnis et al., 1998; Okuno et al., 1998; Drenou et al., 1999; Wolf et al., 1999; Eby et al., 2000; Lankiewicz et al., 2000).

Under certain circumstances, calpain inhibitors blocked all aspects of apoptosis, including DNA fragmentation (Squier et al., 1994; Vanags et al., 1996; Squier and Cohen, 1997). These data imply that calpain activation led to endonuclease activation either directly, or indirectly. However, it is unclear whether this endonuclease activation occurred through activation of a caspase, another protease, or directly by calpains. Others have

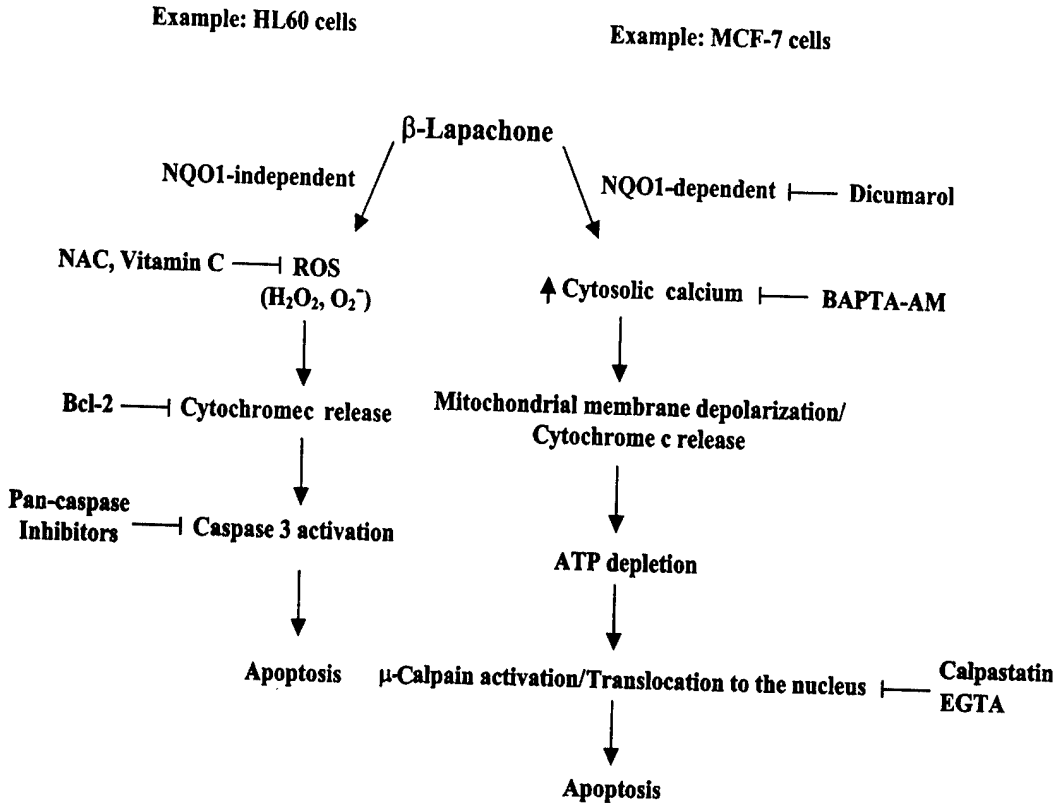

Fig. 3. A model of β-lapachone-induced apoptosis in NQO1-deficient and NQO1-containing cells. Cumulative data indicate that two separate pathways of apoptosis can be induced by β-lapachone. The expression of NQO1 is a key determinant of both the cell death pathway and dose of β-lap required to induce the apoptotic pathway. When cells express NQO1, the cell death pathway stimulated is Ca^{2+}-dependent, blocked by dicumarol and appears to involve the activation of calpain (see text). In the absence of NQO1, cell death appears to occur via a reactive oxygenmediated pathway that is caspase-mediated, and therefore prevented short-term by pan-caspase inhibitors and long tern by over-expression of bcl-2 (see txt). In figure ⊣ or ⊢ represent inhibitory effects, arrows represent stimulatory effects.

demonstrated that calpain inhibitors, but not caspase inhibitors, blocked DNA fragmentation and apoptosis, further implicating a potential role for calpain in endonuclease activation (Squier and Cohen, 1997; Villa et al., 1998). The formation of higher molecular weight DNA fragmentation was inhibited by calpain inhibitors in TNF-induced apoptosis in U937 cells, but was not affected by caspase inhibitors (Vanags et al., 1996). Since calpain inhibitors had no effect on caspase-fluorogenic substrate cleavage, it was suggested that caspases were upstream of calpains and calpain was an effector leading to extra-nuclear and nuclear endpoints (Vanags et al., 1996).

Calpain activation was demonstrated in thymocytes after apoptotic induction by irradiation or glucocorticoid exposures (Squier et al., 1994). Calpain activation preceded apoptotic morphology (DNA fragmentation and nuclear condensation) in dexamethasone-treated thymocytes (Squier and Cohen, 1997). Calpain inhibitors blocked all aspects of dexamethasone-induced cell death, including loss of membrane integrity, decreased cell volume, nuclear collapse and DNA fragmentation (Squier and Cohen, 1997). These data strongly suggest a role for calpains in apoptosis, or their role upstream of caspase activation under certain cellular stress conditions.

Calpains are not only suggested to be involved in DNA fragmentation (via endonuclease activation), but are also effector proteases that cleave cellular proteins involved in DNA repair (e.g., PARP(Buki et al., 1997; McGinnis et al., 1999), membrane associated proteins (e.g., α-spectrin and actin, (Nath et al., 1996b; Villa et al., 1998) and other homeostatic regulatory proteins (e.g., cyclin D1, c-FOS, C-JUN, p53, (Watt and Molloy, 1993; Langenfeld et al., 1997; Pariat et al., 1997; Squier and Cohen, 1997; Carafoli and Molinari, 1998). Calpain substrate cleavage does not involve a specific primary cleavage site, but rather, is dependent upon the secondary structure of the substrate, making calpains a class of proteases different from the aspartate-specific caspases (Croall et al., 1986; Sakai et al., 1987; Wang et al., 1989; Nath et al., 1996b).

Calpains have been implicated in a number of apoptotic pathways. Calpain-like activity in the mitochondria was found to cleave the pro-apoptotic molecule bax. Calpain inhibitors, in turn, inhibited bax cleavage, but did not affect PARP cleavage in HL-60 cells treated with 9-aminocamptothecin, a topoiso-

merase I inhibitor (Wood et al., 1998). Calpains were also involved in calphostin C-induced apoptosis; apoptosis induced by calphostin C in U937 human promonocytic leukemia cells was inhibited by a calpain/proteosome inhibitor, but not by a proteosome-specific inhibitor, further suggesting a role for calpain in apoptosis induced by this agent (Spinedi et al., 1998). Calphostin C is a potent and selective protein kinase C inhibitor in U937 cells (Spinedi et al., 1998).

Both necrotic and neuronal apoptotic cell death were observed in various neurological and neurodegenerative disorders (Wang, 2000). Calpain was activated under various necrotic and apoptotic conditions, while caspase 3 was only activated in neuronal apoptosis (reviewed in Wang, 2000). Despite the difference in cleavagesite specificity, an increasing number of cellular proteins were found to be dually susceptible to both calpains and caspase cysteine proteases. These included alpha- and beta- fodrin, calmodulin-dependent protein kinases, PARP and tau (Nath et al., 1996b; Canu et al., 1998; McGinnis et al., 1998; McGinnis et al., 1999). Neurotoxic challenges such as hypoxia-hypoglycemia, excitotoxin treatment or metabolic inhibition of cultured neurons resulted in activation of both proteases (Nath et al., 1996a; McGinnis et al., 1998; Rami et al., 2000). Calpain inhibitors protected against necrotic neuronal death and to a lesser extent, apoptotic death (Nath et al., 1996a), while caspase inhibitors strongly suppressed apoptotic neuronal death (Nath et al., 1996a). In cell necrosis (e.g., maitotoxin-treated neuroblastoma SH-SY5Y cells) alpha-spectrin breakdown products of 150 kDa and 145 kDa were produced by cellular calpains (Nath et al., 1996b). In contrast, in neuronal cells undergoing apoptosis (cerebellar granule neurons subjected to low potassium and SH-SY5Y cells treated with STS), an additional breakdown product of 120 kDa was observed that was caspase-mediated (Nath et al., 1996b). Inhibition of either caspases or calpains protected both granule neurons and SH-SY5Y cells against apoptosis (Nath et al., 1996b). These data further suggest an interaction between caspases and calpains in apoptosis and neurodegenerative conditions and furthermore, specific intracellular proteins are targeted during cell death processes by both cysteine proteases.

Calpains in β-lapachone-mediated apoptosis

β-Lapachone is a naturally occurring quinone present in the bark of the South American Lapacho tree. The drug has anti-tumor activity against a variety of human cancers, including colon, prostate, promyelocytic leukemia and breast (Li et al., 1995; Planchon et al., 1995; Wuerzberger et al., 1998). β-Lapachone was shown to be an effective agent (alone and in combination with taxol) against human ovarian and prostate xenografts in mice, with low level host toxicity (Li et al., 1999). We recently demonstrated that β-lapachone killed human breast and prostate cancer cells by apoptosis, a cytotoxic response significantly enhanced by NAD(P)H: quinone oxidoreductase (NQO1, E.C. 1.6.99.2) enzymatic activity (Pink et al., 2000a; Planchon et al., 2001). β-Lapachone cytotoxicity was prevented by co-treatment with dicumarol (an NQO1 inhibitor) in NQO1-expressing breast and prostate cancer cells (Pink et al., 2000a; Planchon et al., 2001). NQO1, a cytosolic enzyme elevated in breast cancers (Marin et al., 1997), catalyzes a two-electron reduction of quinones (e.g., β-lapachone, menadione), utilizing either NADH or NADPH as electron donors. We recently demonstrated that reduction of β-lapachone by NQO1 leads to a futile cycling of the compound, wherein the quinone and hydroquinone form a redox cycle with a net concomitant loss of reduced NAD(P)H (Pink et al., 2000a). We also demonstrated that increases in intracellular Ca^{2+} levels were critical for the apoptotic pathway induced by β-lapachone (Tagliarino et al., 2001). Increased cytosolic Ca^{2+}, due to ER Ca^{2+} pool depletion, led to loss of mitochondrial membrane potential, ATP depletion, specific and unique substrate proteolysis, DNA fragmentation and cell death by apoptosis (Tagliarino et al., 2001). Increased cytosolic Ca^{2+} also led to μ-calpain activation and cytoplasm to nucleus translocation that was essential for substrate proteolysis and DNA fragmentation (Tagliarino, et al. unpublished data).

In β-lapachone-treated, NQO1-expressing cells, proteolytic cleavage of PARP correlated well with cell death, as well as classic Lamin B cleavage. However, PARP cleavage did not generate the classic caspase 3/7-mediated 89 kDa fragment. Instead, an ~60 kDa PARP polypeptide fragment was observed. This atypical fragment was most apparent in MCF-7 cells that were more sensitive to β-lapachone, due to elevated NQO1 levels, as well as in prostate cancer cells that over-expressed NQO1 (Pink et al., 2000a; Pink et al., 2000b; Planchon et al., 2001). Atypical PARP cleavage was inhibited by the global cysteine protease inhibitors, iodoacetamide and N-ethylmaleimide (Pink et al., 2000b). Iodoacetamide and N-ethylmaleimide react directly with active site cysteines and thereby inhibit all cysteine proteases, as well as other enzymes that contain accessible SH groups. In contrast, atypical PARP cleavage was insensitive to inhibitors of Granzyme B, caspases, cathepsins B and L, trypsin, and chymotrypsin-like proteases (Pink et al., 2000b). However, extracellular Ca^{2+} chelators, EGTA and EDTA, blocked these proteolytic events in a dose-dependent manner (Pink et al., 2000b). Furthermore, in NQO1-expressing prostate cancer cells, zVAD did not block PARP, p53 or lamin B cleavages after a 4 h pulse of β-lapachone (Planchon et al., 2001). Specifically, caspase 3 was not involved in β-lapachone-mediated cell death, since caspase 3-null and reconstituted MCF-7 cells showed no difference in cell death or proteolytic cleavages, implicating a caspase-

independent apoptotic cell death pathway (Pink et al., 2000b).

Recently, our laboratory demonstrated that β-lapachone-induced activation of μ-calpain in NQO1-expressing human breast cancer cells (Tagliarino, et al. unpublished data). In turn, β-lapachone-induced μ-Calpain activation mediated cleavage of p53 in a manner similar to that previously reported (Kubbutat and Vousden, 1997; Pariat et al., 1997), as well as a novel cleavage of PARP to an ~60 kDa polypeptide fragment. PARP is a caspase 3 substrate, and a widely used indicator of apoptosis when cleaved to a characteristic 89 kDa fragment from its 113 kDa full-length protein. PARP was previously reported to be cleaved by calpains to a 40 kDa fragment during maitotoxin-induced necrosis (McGinnis et al., 1999). PARP was also cleaved by calpains purified from calf thymus to ~42 kDa, ~55 kDa (doublet) and ~67 kDa (triplet) fragments (Buki et al., 1997). We demonstrated that purified μ-calpain cleaved ^{35}S-methionine *in vitro* transcribed and translated PARP to the same ~60 kDa fragment found in extracts from cells treated with β-lapachone, menadione, or ionomycin (Tagliarino, et al. unpublished data). This ~60 kDa PARP cleavage fragment was identical to the fragment observed via Western blot analyses of β-lapachone-treated, NQO1-expressing breast cancer cells (Tagliarino, et al. unpublished data). Calpain activity observed in drug-exposed cell extracts in *in vitro* assays was inhibited by a calpastatin peptide in a dose-dependent manner (Tagliarino, et al. unpublished data). Furthermore, the apoptotic responses in NQO1-expressing cells to β-lapachone were significantly delayed, and survival enhanced, via the exogenous over-expression of calpastatin, a natural calpain inhibitor (Tagliarino, et al. unpublished data).

Upon activation, m-calpain translocated to the nucleus where it could proteolytically cleave PARP and p53 (Tagliarino, et al. unpublished data). We provided evidence that suggests that β-lapachone-induced, μ-calpain stimulated, apoptosis did not involve any of the known caspases; Western blot analyses of β-lapachone-exposed breast cancer cells for apoptotic caspases (3, 6, 7, 8, 9, 10 and 12) demonstrated no change in full-length protein nor did they exhibit formation of the activated proenzyme fragment, 6-24 h after β-lapachone exposures (Tagliarino, et al. unpublished data).

A number of studies regarding the effects of β-lap on cells have also been performed in cells that are deficient in NQO1 (e.g., HL60 cells). In contrast to NQO1-mediated processes elicited by β-lap (discussed above), β-Lap induced caspase-mediated apoptotic responses at higher doses in cells deficient in NQO1. In NQO1-deficient cells, we propose that β-lap undergoes two-one electron reductions to the hydroquinone through a semi-quinone intermediate. This may lead to ROS that would be involved in an NQO1-independent cell death pathway. Chau et al. found a dramatic elevation of H_2O_2 in human leukemia HL-60 cells following 1 μM β-lap treatment and this increase was effectively inhibited with antioxidants *N*-acetyl-L-cysteine (NAC), ascorbic acid, and a-tocopherol (Chau et al., 1998; Shiah et al., 1999). NAC prevented apoptotic characteristics of DNA fragmentation and apoptotic morphology. Over-expression of ectopic bcl-2 in HL-60 cells also attenuated β-lap-induced H_2O_2 and conferred resistance to β-lap-induced cell death (Chau et al., 1998). Studies by Planchon et al. also demonstrated that bcl-2 over-expression in HL-60 cells prevented all aspects of β-lap-mediated cytotoxicity including an enhancement of survival of β-lap-treated cells (Planchon et al., 1999). β-Lap-induced apoptosis in HL-60 cells was accompanied by activation of caspase 3 and classic PARP cleavage that was blocked by caspase-specific inhibitors (Planchon et al., 1999). Over-expression of bcl-2 prevented β-lap-mediated caspase 3 activation and PARP cleavage while increasing viability of bcl-2 expressing cells, compared to vector alone (Planchon et al., 1999).

Therefore, there are two pathways of β-lapachone-mediated apoptosis dependent upon the NQO1 status of the cell type assayed. In β-lapachone-mediated, NQO1-dependent cell death μ-calpain is the primary protease and in NQO1-deficient cells, caspases are the predominant pathway mediating apoptosis. However, we have observed that NQO1-mediated cell death via μ-calpain activation was the dominant cell death pathway following β-lap exposures using genetically matched human breast (Pink et al., 2000a; Tagliarino et al., 2001) or prostate (Planchon et al., 2001) cancer cells.

In conclusion, calpains have been implicated in a number of apoptotic and cell death pathways, however, their pattern of activation as well as their association with caspases in these pathways, remains controversial. Further work on the ability of calpains to activate endonucleases, induce apoptosis independent of caspase activity, and their distinct role in cell death needs further elucidation. More importantly, the caspase- and p53- independent nature of calpain-mediated cell death induced by β-lapachone or various β-lapachone analogs must be further explored to (a) elucidate the unique signal transduction pathway occurring during this unique form of apoptotic cell death; and (b) exploit this cell death pathway for the treatment of human malignances, especially cancers that have lost caspase or p53 functions via accumulated mutations.

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Enhancement of Solubility and Biological activity of β-Lapachone by forming Inclusion Complex with Cyclodextrins

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Abstract: Inclusion complex between β-Lapachone (β-Lap) with four Cyclodextrins including with alpha, beta, gamma and hydroxypropyl-beta-cyclodextrin in aqueous solution has been investigated in order to overcome the solubility problem. The effect of cyclodextrins on the aqueous solubility of β-Lap was evaluated by the phase solubility method. The solubility of β-Lap increased in a linear fashion as a function of alpha, beta and hydroxypropyl-beta-cyclodextrin concentration, and resulting solubility curve can be classified as type A_L. On the other hand, gamma-cyclodextrin showed a typical B_S-type solubility curve. In this study, β-Lap was found to exhibit the fluorescence signal. The excitation wavelength and emission wavelength were found to be 330 and 436 nm. Using fluorescence spectroscopy, 1:1 β-CD-β-Lap inclusion complex was proved. The association constant for the formation of the complex was calculated by phase solubility method and fluorescence spectroscopy. Cytotoxicity through DNA assays indicated the same biological activity of β-CD-β-Lap inclusion complex and β-Lap dissolved in DMSO.

Introduction

β-Lapachone (β-Lap) is a potent cytotoxic agent that demonstrates antitumor activity against a variety of human cancer cells. The drug is bioactivated by the enzyme, NQO1, which is elevated in

numerous cancers, including colon, breast, prostate and lung.[1] Despite its potency and selectivity in killing NQO1-containing cancer cells *in vitro*, the low water solubility of β-Lap (0.04 mg/cc) limits its clinical applications *in vivo* via conventional means of drug administration.

Cyclodextrins (CDs) are well-known host molecules, which can form the inclusion complex with a variety of drugs to improve properties of drugs, such as solubility, stability and bioavailability. [2-8] The α-cyclodextrin (α-CD) comprises six glucopyranose units, β-cyclodextrin (β-CD) comprises seven such units, γ-cyclodextrins (γ-CD) comprises eight such units and hydroxypropyl-beta-cyclodextrin is the modified β-cyclodextrin obtained by treating a base-solubilized solution of β-cyclodextrin with propylene oxide. The entire glucose unit are in the chair conformation linked by α(1,4) glycosidic oxygen bridges, which form a hydrophilic outer surface and a hydrophobic cavity. The hydrophilic outer surface with free hydroxy groups can make it water-soluble. The hydrophobic cavity can enhance the solubility of β-Lap by encapsulation or forming inclusion complexes in aqueous solution. In general, the main driving force for the inclusion complex between a poorly soluble guest and a CD in aqueous solution are the repulsive forces between the included water molecules and the apolar CD cavity and between the bulk water and the apolar guest. [3]

During complex formation, β-Lap will be fit into β-CD cavity diameter (0.78 nm) [2-3] and establishes the dynamic equilibrium with free drug and β-CD molecules in the solution expressed by the complex association constant (K_c). For the formation of a 1:1 complex, K_c and the equilibrium are shown in Fig. 1.

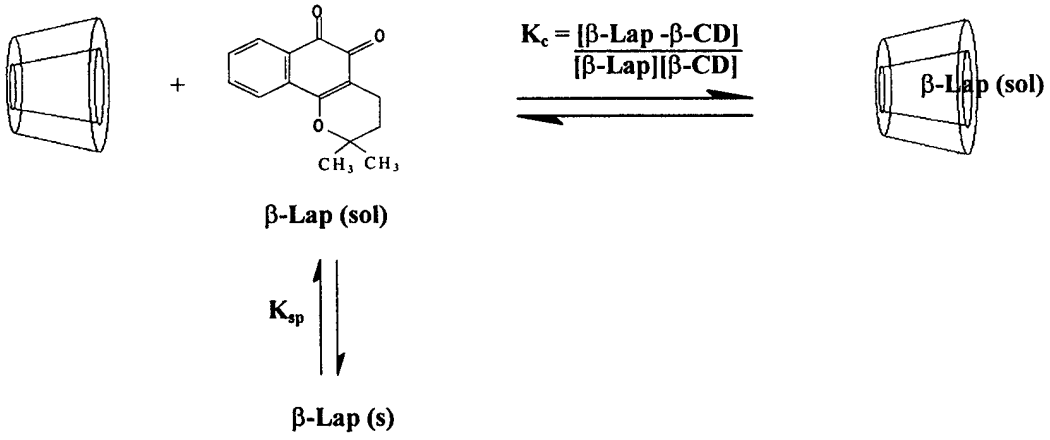

Fig. 1. β-Lap structure and schematic diagram of the equilibrium of β-Lap in aqueous solution in the presence of β-CD where [β-Lap-β-CD] is the concentration of 1:1 β-CD-β-Lap complex, [β-Lap] is the concentration of free β-Lap and [β-CD] is the concentration of free β-CD in the equilibrium.

The aim of this work was to explore the use of Cyclodextrins to form inclusion complex with β-Lap to overcome the solubility problem. The effects of CDs on the aqueous solubility of β-Lap and the interaction of CDs and β-Lap were studied by UV and fluorescence spectrometry, which can provide the association constant (K_c). In addition, the effect of the inclusion complex is evaluated on cell cytotoxicity in DNA assay.

Experimental Section

Materials- alpha, beta, gamma and hydroxypropyl-beta-cyclodextrin were obtained from CTD, Inc., which is over 98% purity. β-Lapachone was obtained from Boothman 's lab. All chemicals were used without further purification.

Dissolution studies of β-CD-β-Lap inclusion complex-Dissolution studies were performed by adding an excess amount of β-Lap (4 mg) in 4 ml of 20 mM potassium phosphate buffer (pH 7.7) containing 0 and 1.58 x 10^{-2} mol/l of β-CD then these solution were stirred at 25 °C. An aliquot was taken at intervals; from its filtrates the amount of β-Lap was determined by UV spectrophotometry (Perkin Elmer instruments Lambda 20) at the wavelength of 257.2 nm.

Phase Solubility studies-Solubility studies were performed by adding an excess of β-Lap in PBS containing different amount of CDs ranging from 0 to the highest solubility of each CD, and stirred at 25 °C until equilibrium. An aliquot was withdrawn, filtered and analyzed for β-Lap by UV spectrophotometry at the wavelength of 257.2 nm. An association constant (K_c) was calculated from the

linear relationship between the dissolved β-Lap and the concentration of CD in the phase solubility diagram by the following equation based on the assumption that 1:1 complex was formed. [19]

$$K_{1:1} = \frac{\text{slope}}{\text{intercept x (1-slope)}} \quad (1)$$

Fluorescence study of β-CD-β-Lap inclusion complex- In order to obtain the correct excitation and emission wavelength, the emission spectra of β-Lap were preformed by LS 45 Luminescence Spectrometer from Perkin Elmer instruments with 100 nm/min for the scan speed and 10 nm for both excitation slit width and emission slit width.

Fluorescence study of complex-A stock solution of β-Lap 4 x 10^{-6} mol/l in PBS was prepared. Different amounts of β-CD were dissolved in this β-Lap stock solution to get 1.54 x 10^{-4} mol/l to 1.58 x 10^{-2} mol/l of β-CD. The solution was vigorously stirred at 25 °C overnight until equilibrium before measurement. Every spectrum used β-CD blank for background subtraction.

Cytotoxicity assay-The cytoxicity of β-CD-β-Lap inclusion complex was studies by DNA assay. X x 10^{x} cells/mL of XXX cells were seeded in the 48-well plates (**Fisher ?**) and then incubated for X h in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were wash X times with PBS solution and then incubated for x h in a humidified atmosphere 5% CO_2 at 37 °C with XXX culture medium with the different concentrations ranging from 0-X μM of β-CD-β-Lap inclusion complex. In order to study the effect of pure β-CD on cell growth, control experiments with the same amount of β-CD in the 0 to X μM range were carried out. The same experimental setup was prepared by using β-Lap dissolved in DMSO in the same concentration as β-Lap-β-CD complex. After washing X times with PBS to remove β-CD, cells were fixed with XXX and submitted to alkaline hydrolysis (0.x M XXX). The absorbance of the XXX was recorded at xxx nm in order to measure nucleic acid.

Results and Discussion

Dissolution study- The results showed that the amount of β-Lap dissolved from β-CD containing solution is higher than β-Lap alone at each time point. It takes at least 24 h to reach the equilibrium.

Solubility study- The phase solubility diagram of β-Lap with β-CD is shown in Fig. 2.

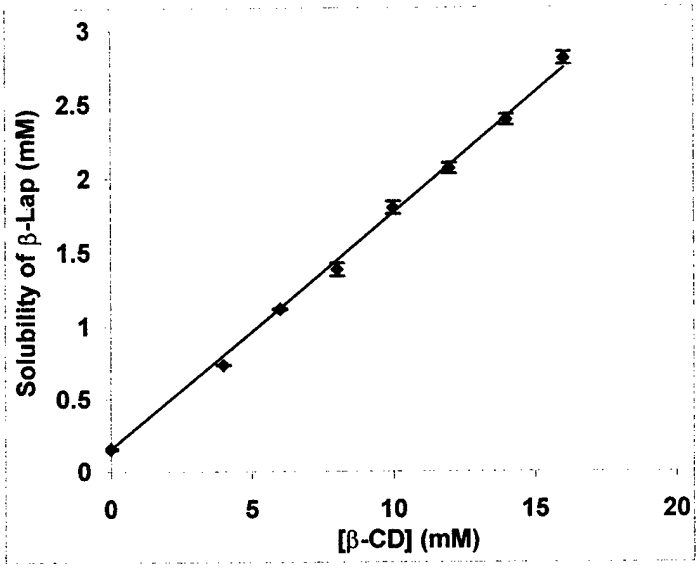

Fig. 2. Solubility of β-Lap as a function of β-CD concentration at 25 °C

It was found that increasing in β-CD molar concentration linearly enhanced β-Lap solubility and can be classified as type A_L. [19] This is because β-Lap having very low solubility in water goes into the hydrophobic cavity of β-CD. In the other word, this indicates the formation of the inclusion complex between β-CD and β-Lap. The solubility of β-Lap increased about tenfold (from 0.16×10^{-3} to 2.80×10^{-3} mol/l) in solution containing 0.0158 mol/l β-CD, which is the maximum solubility of β-CD. The association constant calculated from equation 1 assuming the formation of a complex with a 1:1 complex is $1.28 \pm 0.3 \times 10^{3}$ M^{-1}. However, the addition of β-CD did not significant change the absorption spectra of β-Lap.

Fluorescence study

Emission spectrum of β-Lap is shown in Fig. 3. The emission bands were constant at 436 nm when the excitation wavelengths were changed from 257 to 360 nm. It was also found that the excitation wavelength at 330 nm gives the highest emission intensity.

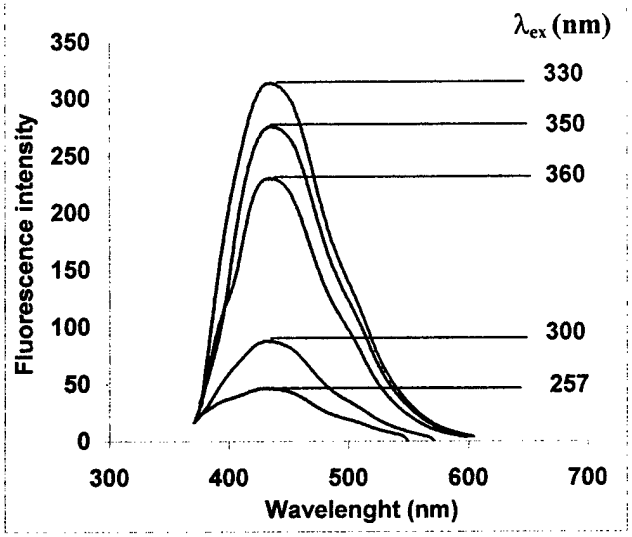

Fig. 3. Emission spectra of β-Lap at various excitation wavelengths

The effect of the concentration of β-CD on the fluorescence spectra obtained by exciting at 330 nm is shown in Fig. 4. The remarkably quenched of the emission intensity and the blue shift around 6 nm of the emission maximum were found. The blue shift indicates β-Lap experience a less polar environment causing by the hydrophobic cavity of β-CD.

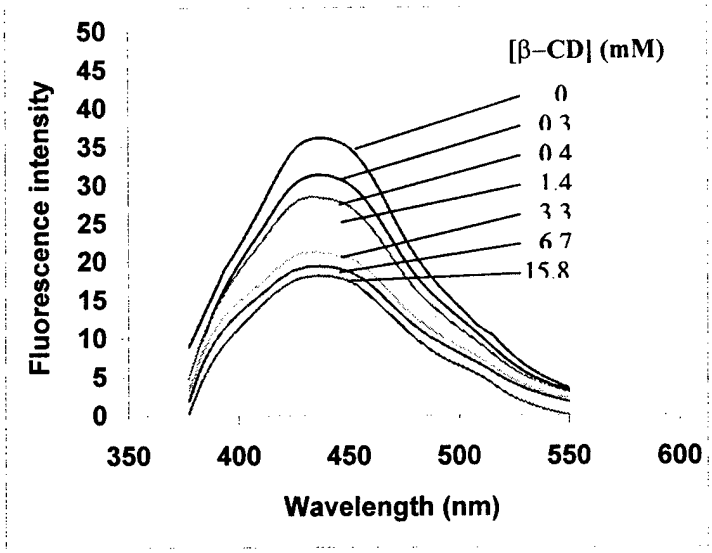

Fig. 4. Emission spectra of β-Lap (4×10^{-6} mol/l) in various β-CD concentrations at 25 °C

The plot shows the emission intensity decreased with increasing β-CD concentration. The change of fluorescence intensity of β-Lap (increase [10-14] or decrease [14-18]) by the addition of β-CD is another indication of the formation of the inclusion complex between β-CD and β-Lap. Considering that there are two fluorescence substances in the system, the free drug (S) and the complexed drug (SL) having lower fluorescence intensity than the free drug, the total fluorescence intensity, which decreases as β-CD (L) increases and then reaching a constant value, is the sum of the contribution of these two substances. In this kind of system, the molar ratio (R) of free drug and complexed drug at each β-CD concentration can be demonstrated by the equations 2 and 3, respectively.

$$R_S = (F_o - F_T) - (F_o - F_i) \ / (F_o - F_T) \quad (2)$$

$$R_{SL} = (F_o - F_i) \ / (F_o - F_T) \quad (3)$$

where F_o and F_T denote the fluorescence intensity of β-Lap in the absence and in the presence of excess β-CD, F_i stands for the total fluorescence intensity of the β-Lap in β-CD

Assuming the formation of the 1:1 β-CD-β-Lap inclusion complex, the concentration of S and SL in the association constant as shown in fig. 1 can be replaced by R_S and R_{SL}, respectively resulting a scatchard equation (2) as shown below.

$$R_{SL} / [\beta\text{-CD}] = -R_{SL} K_{1:1} + K_{1:1} \quad (2)$$

The rearrangement of equation 2 provides equation 3, which can be used to represent and determine the association constant.

$$(F_o - F_i) / [\beta\text{-CD}] = -(F_o - F_i)K_{1:1} + (F_o - F_T)K_{1:1} \quad (3)$$

The plot between $(F_o\text{-}F_i) / [\beta\text{-CD}]$ versus $(F_o\text{-}F_i)$ shown in Fig. 5 exhibit a linear relationship for the whole range of β-CD concentration indicating the agreement with 1:1 complex assumption in derivation of equation 2. A $K_{1:1}$ value of $1.10 \pm 0.09 \times 10^3$ M^{-1} is evaluated from the ratio of intercept/slope, which closes to a $K_{1:1}$ value calculated from the phase solubility method ($1.28 \pm 0.03 \times 10^3$ M^{-1}).

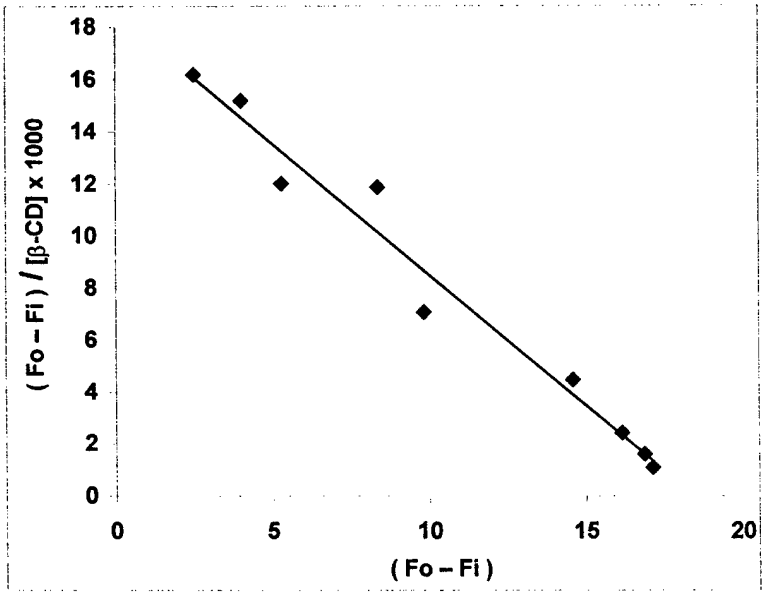

Fig. 5. Plot of (F_o-F_i) / [β-CD] versus (F_o-F_i) obtained from β-Lap (4×10^{-6} mol/l) in various β-CD concentrations at 25 °C

DNA assay

The Cellular growth of X cells exposed to different concentrations of β-CD-β-Lap inclusion complex, β-Lap dissolved in DMSO and pure β-CD were shown in Fig. 6.

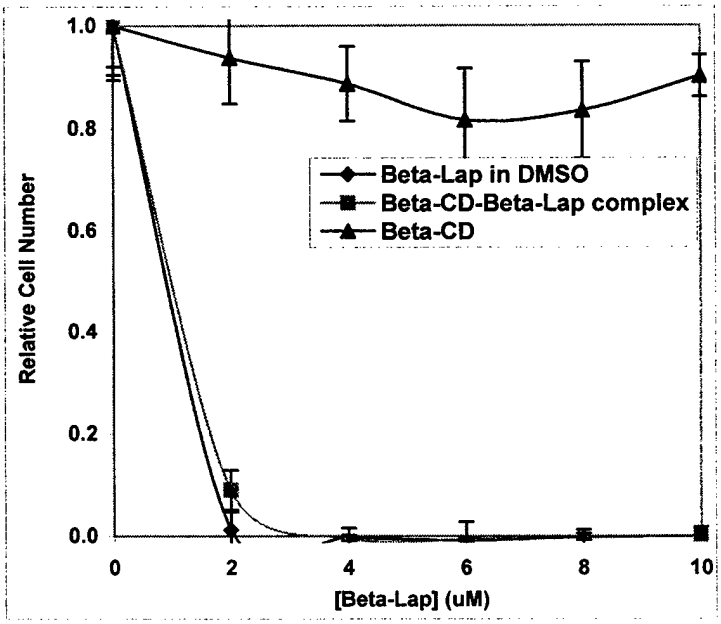

Fig. 6. Cells viability in the presence of β-CD, β-CD-β-Lap inclusion complex and β-Lap/DMSO

The dose-response in the DNA content was x μM and x μM for β-CD-β-Lap inclusion complex and β-Lap dissolved in DMSO, respectively. The entire dose of both β-CD-β-Lap inclusion complex and β-Lap dissolved in DMSO showed **almost** the same response. The control experiments with the pure β-CD in 0 to X μM range showed no cytotoxicity up to X μM. This indicates that the activity of β-CD-β-Lap inclusion complex and β-Lap dissolved in DMSO were the same. And it also showed the success of improvement of β-Lap solubility by β-CD and the formation of the inclusion complex between β-CD and β-Lap.

Conclusions

In this study, β-Lap was successfully formed the 1:1 inclusion complex with β-CD resulting a significant enhancement of β-Lap solubility. Phase solubility and fluorescence spectroscopy showed the important data for calculating the association constant of the 1:1 β-Lap-β-CD inclusion complex. The biological activity of β-CD-β-Lap inclusion complex and β-Lap dissolved in DMSO studied by DNA assay showed the same response.

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